THE CONTROL OF CELL DIVISION DURING THE IMMUNE RESPONSE

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A Summary.

Following immunization with antigen a proliferative response was detected in both the bone marrow and spleen. Concomitant with this heightened proliferative activity there occurred a significant hypercalcaemia. The elevated proliferation was always associated with a hypercalcaemic episode regardless of the antigen chosen. Removal of the parathyroid glands causes severe hypocalcaemia and under these conditions the thymus becomes severely involuted and the bone marrow hypoplastic. No incremental increase in plasma calcium occurred in aparathyroid rats following antigen nor was there any proliferative response in the bone marrow. Aparathyroid rats rendered normocalcaemic by feeding a high calcium/low phosphate diet likewise failed to show any proliferative response in the bone marrow following antigenic challenge. The proliferative response of splenic lymphocytes in response to antigen was largely unaffected by parathyroidectomy. The immune response of rats was impaired by parathyroidectomy. Primary antibody responses to heterologous erythrocytes were slightly lower than normal animals as were the numbers of haemolytic plaques which develop in the spleen. Parathyroidectomy potentiated the delayed-type hypersensitivity response to oxazolone, perhaps suggesting a selective deleterious effect on a suppressor-cell population. Splenic lymphocytes from parathyroidectomized animals were able to elicit a normal graft--versus-host reaction as assessed using a popliteal lymph node weight assay. It was also shown that a normal pool of memory cells was formed by aparathyroid rats. However, reduced hyperplasia of the node occurred when the recipient rat was parathyroidectomized. In view of the differential effect which parathyroidectomy had on the proliferative response of the bone marrow and spleen, it seems likely that the target cells for parathyroid-mediated enhancement of proliferation are antigen-insensitive. It is postulated that parathyroid hormone may have a rôle in the replenishment of the lymphoid cell pool by stimulating increased division of more primitive lymphoid elements.

Key Words: Calcium; Cell Division; Immune Response; Parathyroid Hormone.

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Section 1.

Introduction.

1.1 General Introduction.

Protozoa clearly perform all the functions necessary for the maintainance of life including recognition of substances as either beneficial or harmful and produce appropriate responses to those agents. Thus a food substance may be engulfed and digested while noxious substances may be avoided or engulfed and either destroyed or encapsulated before expulsion from the cell. During metazoal organization this multiplicity of function has been progressively lost as groups of cells formed tissues with more or less specialized function. In higher animals the resistance to infection is largely maintained by the cells and tissues of the immune system. The primary function of the immune system is to remove foreign substances from the body and to this end some elements have become super--specialized at recognizing 'non-self' from 'self' while others perform the phagocytic function found in unicellular organisms.

The fundamental capacity of cells to respond to stimuli has been exploited many times in the development of a multicellular organization. Cells have become grouped into sensors and effectors of internal change and serve to maintain the "milieu interieur" constant thus giving the organism tremendous independence from the external environment. Any chemical or physical change of the internal environment is usually counteracted by negative feedback loops which establish a state of physico-chemical equilibrium. In its broadest sense, the stability of the internal environment demands freedom from infectious organisms and their by-products, which constitute a deviation from constancy. It falls upon the specialized cells and tissues of the immune system firstly to detect the presence of a foreign substance, and secondly to elicit those responses necessary for its elimination. Thus just as an increase

in (say) osmotic pressure stimulates antidiuretic hormone release to re-establish normality so intrusion of foreign substance likewise provokes rapid restorative reactions from the immune system.

Not only is there a constancy in the composition of the extra--cellular fluid in which the cells and tissues are bathed there must also be a constancy of cell number in these tissues to ensure their normal function. This is particularly so in constant renewal tissues where feedback devices must exist to ensure that cellular production balances cellular elimination. The capacity for a controlled elevation of cell production in response to a functional demand must also be retained. Thus in the immune system, mechanisms exist which equate cellular attrition with generation. In addition, one cardinal feature of an animal's response to foreign bodies is the rapid clonal expansion of the numbers of cells capable of recognizing and reacting to this intrusion to bring about its more rapid elimination.

All cells which possess a capacity to proliferate are inevitably subjected to some form of regulation of division no matter how crude. Clearly, with tumour cells, any proliferative control is hopelessly inadequate, but even these cells experience factors which limit their rate of proliferation. In the healthy body, cells are organized into tissues with fundamental differences in cellular kinetics and which exhibit several different types of proliferative control mechanisms. The introduction to this thesis will firstly review what is known of the biochemistry and physiology of cell division and its control in eukaryotes. Emphasis will be placed on mammalian cells and in particular, lymphoid cells. The lymphocyte is one member of a group of cells involved in the immune response and has its own specialized regulation system in addition to more fundamental regulators which are common to all proliferating cells. The second part of the introduction contains an account of the immune system and of the

proliferative control to which immunocytes are subjected. The theme which will be developed is that hormonal homeostatic systems are not only involved in creating ambient conditions favourable to optimum expression of immunity, but may also act as a driving force in the lympho-proliferative events of the immune response.

1.2 The Cell Life Cycle.

The cell life cycle includes all the events which occur between a cell's 'birth' and death in contrast to the Mitotic Cycle which refers only to the period between two consecutive divisions. The life-span of a particular cell and the number of times that it divides within that period determines the size of clone possible from an individual cell. Within a population of cells, slight changes in either life span or in cell cycle time will have profound effects on the size of that population. It follows that a scrutiny of the factors affecting the cell cycle is essential before the clonal expansion of a previously quiescent population of lymphocytes can be appreciated.

The most obvious feature to be seen in the life of a eukaryotic cell is when it divides to form two daughter cells. This event, termed mitosis, was seen by the early microscopists to contain several morphological stages. Thus the chromosomes became visible as tightly coiled, but discrete structures within the nucleus whose membrane began to disappear (prophase); they then became attached as an obviously double-stranded structure to a central or equatorial plate (metaphase); the two halves of this doublet split and moved to opposite ends of the cell (anaphase); a new nuclear membrane formed around the two groups of daughter chromosomes while the cytoplasm was pinched off between them such that a discrete plasma membrane formed around the new nuclei and cytoplasm (telophase). The chromosomes then adopted a less discrete form and could only be seen as diffuse strands or clumps of nuclear material or chromatin and remained essentially thus until another mitotic sequence was initiated. This period between two mitotic events is termed interphase. Considering the major reorganization which occurs during mitosis it is

superficially surprising that it occupies a relatively tiny part of the cell's life cycle. The apparent quiescence of the cells in the relatively long interphase, when viewed by light or electron microscopy, belied the true complexity of the biochemical events occuring during this phase.

The formation of two daughter cells each with the same genetic complement, or genome, as the original parent cell, demands the replication of DNA. It is during interphase that this occurs. Howard & Pelc (1953) using autoradiography, demonstrated that DNA synthesis in Vicia faba root cells was not continuous but occurred in a discrete period of interphase which they termed the S or synthetic phase. This was preceeded and followed by gaps (G_1 and G_2 respectively). Thus for a cell undergoing continued rounds of mitosis (M) the sequence M, G_1, S, G_2, M .... etc was observed and is referred to as the Mitotic Cycle (fig.1.1). Cells in G_1 have 2n complement of DNA, those in G_2 have 4n, while cells in S range from 2n to 4n. Though a 'typical' cell cannot be represented, many mammalian cells in culture average 8-10 hours for G_1, 6-7 hours for S, have a G_2 of 3-5 hours and spend about 1 hour in mitosis giving a total cycle time of 18-20 hours (Mitchison 1971). The majority of eukaryote cells conform to this general mitotic cycle though exceptions have been found in rapidly dividing tissues such as early embryos or in Ascites tumour cells which can lack a G_1 (Ord,1973).

It has become evident that the mitotic cycle of Howard and Pelc does not include all the events of the cell life cycle. In some cell populations there is an apparent lack of any mitotic event e.g. nerve cells, which after their genesis from neuroblasts never divide again. In others there may be an alternation of resting periods and periods of active proliferation. Different patterns of organization provide conditions necessary for population size control, cell differentiation,

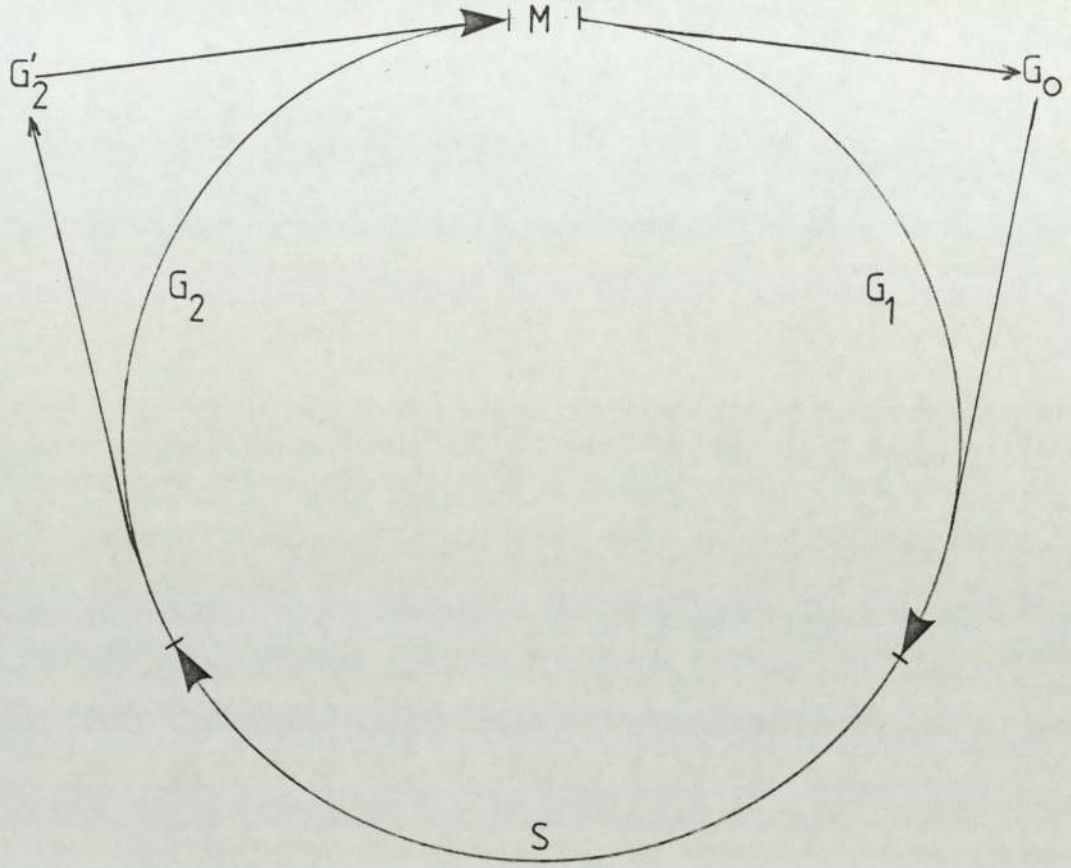


Figure 1.1 The mitotic cycle of a typical cell. Following mitosis (M) the daughter cells may either pass into a pre-DNA synthetic phase G_1 or into an extended quiescent phase (G_o). Most circulating peripheral lymphocytes are in G_o and can be rapidly recruited into the DNA-synthetic (S) phase of the cycle. Once committed to S most cells complete the cycle through G_2 (when the cells have a 4n complement of DNA) and into mitosis. In some tissues, notably the ear epithelium of rabbit, cells are apparently able to enter M almost directly from a suspended G_2 condition. These cells are said to have been in G_2' phase.

interaction of a given population with other systems and reactions of cells to a changing environment. When stimulated, cells in resting states may enter the mitotic cycle, divide and differentiate. However, the completion of mitosis itself does not imply that a cell will undergo differentiation, for in the absence of adequate stimulus it may pass again into a resting period (Epifanova & Terskikh,1969). This quiescent population of cells, designated as being in a G_o phase (Lajtha,1963; Quastler,1963), are thus mitotically competent and can be recruited into the mitotic cycle, probably at the G_1/S boundary (Whitfield, Perris & Youdale,1969a; Whitfield, Rixon, Perris & Youdale,1969b). A G_o phase has been shown to exist in cells of many tissues notably bone marrow (Lahiri & Van Putten,1972), some tumour cells (Wilson & Gehan,1970), chick intestinal epithelium (Cameron & Ceftman,1964) and in blood borne lymphocytes (Knight & Ling,1968). Another resting period G_2' occurs in some tissues such as rabbit ear epithelium (Gelfant,1962). Here some cells remain quiescent after the completion of DNA synthesis and can be recruited directly and rapidly into M on receipt of an appropriate stimulus such as wounding.

The cells of different tissues may in fact be described in part on the basis of their proliferative characteristics (see fig.1.2). Thus neuronal cells (type A) show no proliferative activity and are described as static end cells. Only when cell death occurs do cells leave the population; no cells leave the neuronal compartment to differentiate and become another type. Other populations of cells (type B) may be transit stages between one type and another (e.g. reticulocytes) and provided the rate of entry and exit from this transit pool are equal, cell numbers remain constant. Some populations e.g. ovary cells (type C) may slowly decline because there is no renewal via cell division. It is also possible that cells within a compartment may have the ability to proliferate

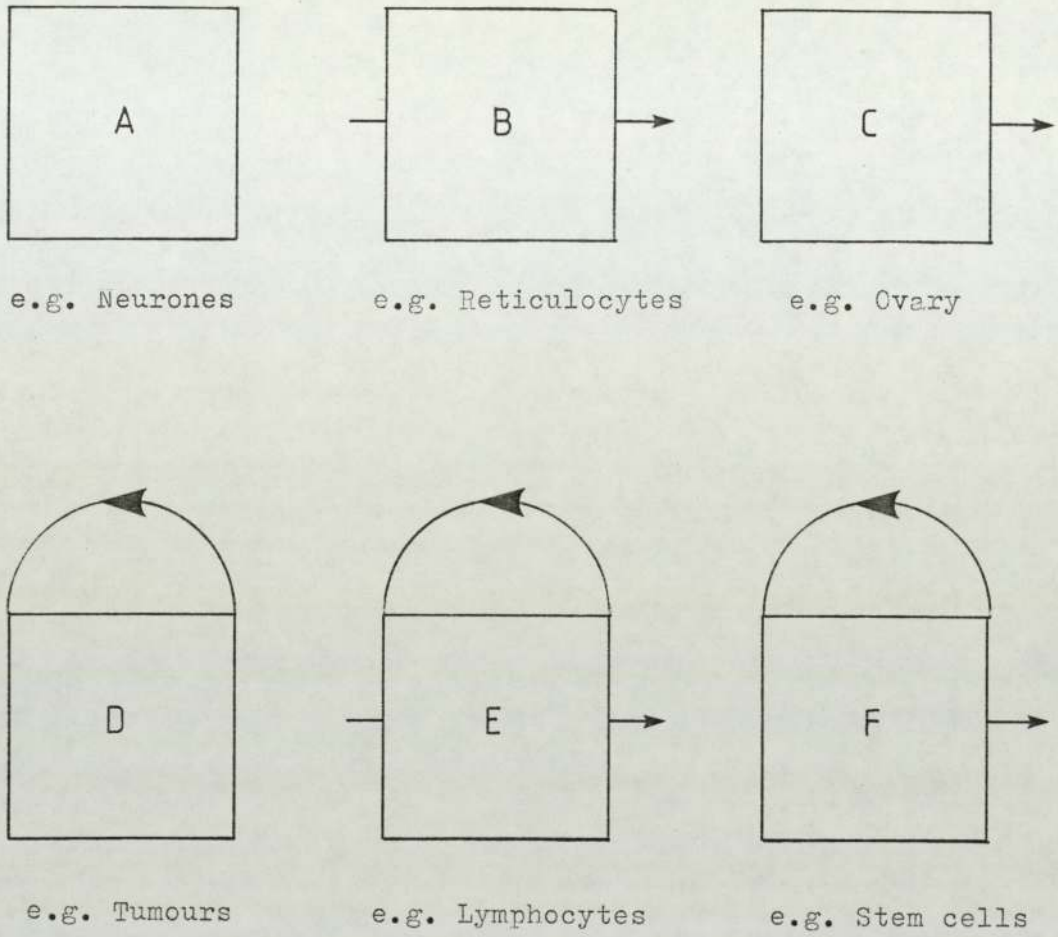


Figure 1.2 A schematic representation of theoretical cell populations. A represent a static cell population such as neurones. B is a transit cell population such as reticulocytes while C is a cell population in decline which might be found in the ovary. The cell-types A-C lack the capacity for cell division. The introduction of this feature gives rise to cell populations with entirely different characteristics. Thus cell type D is a closed, dividing population which may occur in tumours or in regenerating liver. Type E represents a dividing transit population such as activated lymphocytes. Finally type F represents a dividing pool of cells with in input of cells and is thus the stem cell compartment.

without differentiation. The size of such cell pools is thus determined not only by the rate of entry to, and exit from the pool, but also by the proliferative activity of the cells with that population. Thus a closed, dividing population of cells e.g. tumours, regenerating liver (see fig.1.2; type D) has no cells leaving the compartment (other than by cell death) and no cells enter from another population. The size of such a population of cells is determined totally on the rates of cell division and cell death; if cell division exceeds cell death the population will expand. Many of the cells of the bone marrow, other than stem-cells and lymphoblasts in secondary lymphoid tissues, are members of transit cell populations which can also proliferate (type E). Such populations are important in providing the tremendous output of cells possible during erythropoiesis or lymphopoiesis which results from amplification steps along the maturation pathway. Finally the stem cell compartment (type F) is a cell population with a capacity for self renewal, by mitosis, which is not replenished from another population but cells leave to differentiate into different cell types.

Cleaver (1967) has combined these concepts of dividing, quiescent, maturing and differentiating cells to provide a generalized four compartment model of cell renewal (fig.1.3) which represents schematically the relationships between population of cells within a tissue. The model has particular relevance to the cells of the immune system which show a retained capacity for sudden proliferative activity by cells recruited from G_o and replacement of cells from a stem cell compartment in the bone marrow. In many tissues in the adult some of these compartments, particularly the stem cell compartment, may cease to exist. However, the remaining proliferative cell populations have many potential sites at which DNA synthesis can be controlled resulting in expansion or depletion

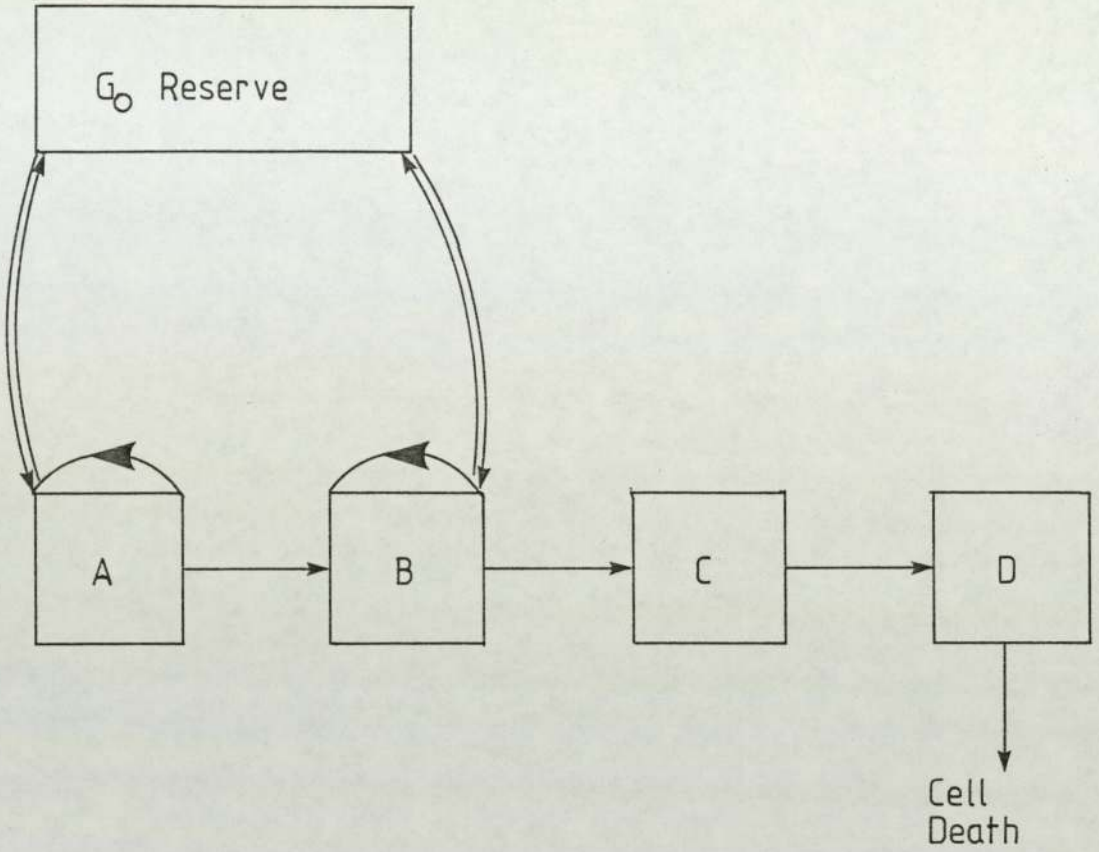


Figure 1.3 A four-compartment model for cell renewal which consists of a self maintaining stem cell compartment (e.g. bone marrow), B; a maturation pool containing dividing cells (e.g. thymus) C; Maturation pool without dividing cells (e.g. reticulocytes) D; Mature functional compartment (e.g. polymorph, plasma cell). The G_o reserve consists of cells which are indistinguishable morphologically from the cells in either A or B and are thus shown together. These cells may enter the cycling pools when the need arises. (After Cleaver, 1967).

of both that cell population and its descendants. In lymphocytes, an initial membrane stimulus provided by a large, non-soluble antigen (e.g. foreign erythrocytes) may be sufficient stimulus for the cell to enter S. However, in the final analysis cell division is essentially an intracellular event and it was within the cell that control sites were sought. Demonstration that the cytoplasm was involved in the entry of a cell into S was achieved by showing that inhibition of protein synthesis by either puromycin or cycloheximide prevented, or substantially reduced, the numbers of cells entering S (Block, MacQuigg, Brack & Wu,1967) yet once committed to S, susceptibility is reduced (Kim, Gelbard & Parez,1968). It thus appears that there is at least one point, late in G_1, where cells "make a decision" to enter S and having done so complete the cell cycle up to the formation of two daughter cells both in G_1 (Frankfurt,1968). DNA synthesis, around which the Howard & Pelc model rotates, is not in reality a single phase but consists of a number of merging enzymatic steps so that the spectrum of enzyme activity changes throughout the cycle. Much earlier work centred on studies of enzyme activity at various stages of the cycle and is worthy of consideration here because it must be possible to demonstrate that any putative DNA synthetic control mechanism can directly or indirectly effect the enzymes responsible for DNA synthesis. Bacteria have been a popular choice for this work, largely because they are amenable to batch culture which allows bulk estimation of enzyme activity and can be readily synchronized by one of several methods so that for a few rounds of division most of the cells are in the same phase of the Mitotic Cycle. Using synchronized cultures of Escherishia coli it was found that of those enzymes showing intermittent activity there were two distinct types. Some enzymes suddenly became active at a point in the cycle

remaining so for the rest of that cycle and were called 'step enzyes' while others showed a pulse of activity and were termed 'peak enzymes'. It should be remembered that many workers equated the appearance of enzyme activity with enzyme synthesis whereas it is possible, even likely, that some enzymes may have already been synthesized in an inactive form and are activated by a co-factor. The inhibition of enzyme activity by the reaction product may, given the correct constants, provide oscillatory enzyme activity of the type observed during the cell cycle. Alternatively, transcription of the DNA may be linear, resulting in enzyme activity appearing in the same sequence as their code on the DNA, or a combination of linear reading with oscillatory repression may occur. While these simple models, originally constructed for prokaryotes, may have some relevance in eukaryotic cells, they clearly give an incomplete picture of intracellular control mechanisms. One of the earliest events following stimulation is the uptake by the cell of a variety of small molecules many of which are not only raw materials for the immenent synthesis but also act as regulators in their own right through enzyme repression and inhibition. The circulating lymphocyte, for example, responds to an external antigenic stimulus by leaving the G_o compartment and entering into DNA synthesis. The observation that a membranal stimulus can lead to a nuclear response resulted in the suggestion that special transmitter substances might exist conveying information from the membrane to the nucleus. The next section reviews the current status of a group of compounds, namely the cyclic nucleotides, which are considered to be potential regulators of cellular proliferation by acting in a 'secondary messenger' rôle, relaying membranal stimuli to sites in the cytoplasm or nucleus.

1.3 The Rôle of Cyclic Nucleotides and Ions in the Control of Cell Division.

In cells which are not undergoing continued rounds of division, but rather are quiescent, the first control point lies in the cell's activation. The stimulus is often but not always, membranal and may be sufficient to precipitate one or several rounds of division. This implies the existence of a mechanism for recognition of the stimulus and its transduction into a chemical signal, or second messenger, which can be transmitted to the site within the cell where it causes a response (fig.1.4). Thus the second messenger is generated by the interaction of an external stimulus with a cell recepter and passes into one or more subcellular compartments where it initiates the appropriate response. The second messenger may also be a critical regulator of the response which it initiates, but it is possible for a substance to be a critical regulator of the cellular response without being a second messenger. Thus generation of secondary messenger does not automatically result in a response from the cell for there are often other regulatory components which must be present before the reaction can proceed. The idea that all cells respond to different stimuli by producing the same second messenger was a tempting one, and the discovery of cyclic 3', 5'-adenosine monophosphate (cAMP) and its rôle in the action of epinephrine on hepatic glycogenolysis (Sutherland & Rall, 1958) elected this nucleotide as prime candidate for the ubiquitous second messenger in cell activation.

The concentration of intracellular cAMP is a function of its synthesis, catalysed by the membrane-bound enzyme adenylate cyclase, its degradation to 5' AMP via phosphodiesterase and leakage of the nucleotide from the cell into the surrounding tissue fluid or medium.

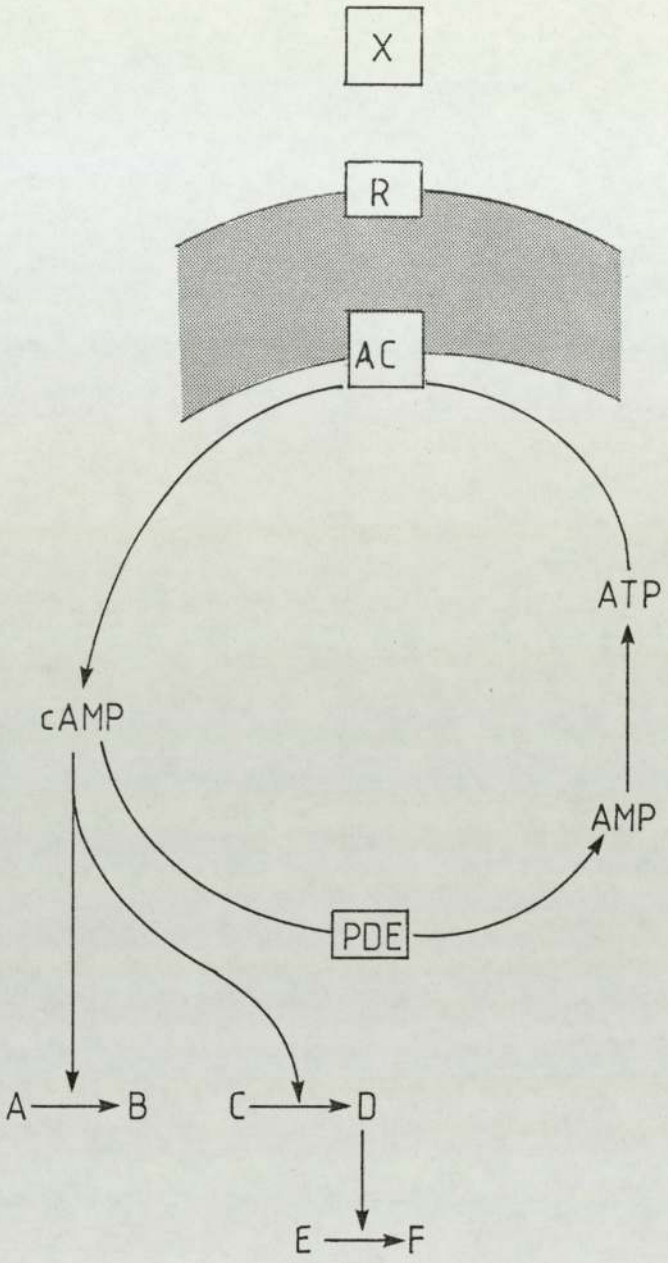


Figure 1.4 A schematic representation of the original second messenger hypothesis in which hormone (X) interacts with receptor (R) which somehow results in stimulation of membrane-bound adenylate cyclase (AC). This enzyme catalyses the synthesis of cAMP, the concentration of which rises. This may lead to the direct catalysis of reaction A--B or may promote the production of D which in turn is required for a key reaction E--F. The degradation of cAMP to AMP is controlled by the enzyme phospodiesterase (PDE). The system may also be represented as a four-step information transfer system; recognition, transduction, transmission, response (adapted from Rasmussen & Goodman, 1977).

It follows that either stimulation of adenylate cyclase or inhibition of phosphodiesterase results in elevated intracellular cAMP concentrations. Work on clarification of the role of cAMP as second messenger in polypeptide hormone action was aided by a number of technical factors. A protein binding assay (Gilman,1970) was available to measure intracellular cAMP content; it was possible to alter its intracellular concentration by use of drugs such as theophylline and imidazole (Robison, Butcher & Sutherland,1971; Sutherland, Robison & Butcher,1968); and it was possible to prepare stable synthetic analogues such as dibutyryl cAMP (DBcAMP) that, when added to the extracellular medium, would initiate intracellular responses in a variety of tissues. In fact, it was these properties which allowed the development of certain criteria for establishing a role of cAMP as a second messenger (Sutherland, et al.,1968). Though originally designed for cAMP in the mechanism of hormone action, with slight modifications these criteria are equally applicable to other tissues and potential second messengers. Cyclic AMP was considered of second messenger status if:

(a) Application of first messenger to the cell led to a rise in intracellular cAMP concentration.

(b) Inhibitors of phosphodiesterase, the enzyme catalysing the hydrolysis of cAMP to AMP, enhanced the physiological effect of sub-maximal concentrations of the first messenger.

(c) Application of exogenous cAMP mimicked the physiological effect of the first messenger.

(d) A first messenger-sensitive adenylate cyclase could be identified in a presumptive plasma membrane fraction of the cell homogenate.

Interest in cAMP as a regulator of cell division arose when it was noted that polyoma-transformed fibroblasts from baby hamster

kidney tissue (BHK cells) contained reduced levels of cAMP. Normal fibroblasts grown in culture entered a quiescent phase with very little proliferation either if starved of a serum supplement or if the density of the cells became too high when the cells were said to exhibit density-dependent inhibition of growth (DDI) (Stoker & Rubin,1967). These arrested populations could be stimulated to reinitiate active proliferation either by the addition of serum or by re-seeding at a lower density. As BHK cells approached confluency, cAMP levels rose (Otten, Johnson & Patten,1971; Bannai & Sheppard,1974) and there existed a neat, inverse relationship between cellular cAMP content and proliferation rate (Otten, et al., 1971). If cAMP was added to the medium of BHK cells which were in the logrithmic phase of growth, the proliferative activity was greatly reduced (Burk,1968). Further, if endogenous cAMP was raised either by inhibition of phosphodiesterase with caffeine or theophylline (Burke,1968) or stimulation of adenylate cyclase with prostaglandin E_1 (Johnson & Pastan,1971) cholera toxin (Pastan, Johnson & Anderson,1975) or bacterial glycolipid (Brailovsky, Trudel, Lallier & Nigram,1973) then proliferation was invariably reduced. Thus an antiproliferative action was ascribed to cAMP. When some tumour cell lines were found to have reduced intracellular cAMP concentrations (Pastan & Johnson,1974; Ryan & Heidrick,1974) the potential therapeutic value of this cyclic nucleotide for cancer was obvious. Following addition of cAMP to the culture medium, an increase in intracellular cAMP is not usually detectable. This is partly due to the poor incorporation of the nucleotide and its rapid breakdown to AMP, ADP, ATP and adenosine (Hsie, Kawashima, O'Neill & Shröder,1975). Since cAMP was not absorbed readily into cells it often proved necessary to use rather large extra cellular doses but the subsequent discovery of analogues, such as DBcAMP, alleviated

this problem as they were easily absorbed, resisted degradation and showed the familiar inhibition of fibroblast growth (Sheppard 1971, Burger, Bombik, Breckenridge & Sheppard 1972; Carchman, Johnson, Pastan & Scolnick,1974; Froelich & Rachmeler,1974). However, technical problems make interpretation of data obtained with DBcAMP difficult. External DBcAMP both inhibited and acted as substrate for phosphodiesterases so that a range of butyrated compounds accumulated in the cell (Kaukel, Fuhrmann & Hilz, Kaukel,1972a; Hsie, et al.,1975; O'Neill, Schröder & Hsie,1975) which may prevent DBcAMP from acting in a totally analogous fashion to cAMP.

Another cyclic nucleotide, cyclic 3', 5'-guanosine monophosphate (cGMP) is also present in cells but at about one tenth the concentration of cAMP. Work done on this cyclic nucleotide presented the impression that it was directly antagonistic to cAMP. Thus in complete contrast to cAMP, cGMP was found in lower concentration in density-inhibited fibroblasts (Moens, Vokaer & Kram,1975) and the addition of serum to cultures which were density-inhibited (Moens et al.,1975) or to serum starved cells (Seifert & Rudland,1974a) resulted in a rapid increase in cGMP preceeding the onset of DNA synthesis. A fibroblast growth factor which, like serum, also reinitiated proliferation in arrested 3T3 cultures similarly elevated endogenous cGMP in these cells (Rudland, Gospodarowicz & Seifert, 1974) by stimulating guanylate cyclase (Rudland, Hamilton, Hamilton, 1975). From observations such as these came the proposal that cAMP and cGMP were antagonistic intracellular second messengers, concerned with retarding and initiating cell division respectively (Golberg, Haddon, Estenstein, et al.,1973; Golberg, O'Dea & Haddox,1973b; Golberg, Haddox, Dunham, Lopez & Hadden,1974; Golberg, Haddox, Nicol, et al.,1975). The hypothesis, termed the yin-yang hypothesis, suggests that in conditions where intracellular cAMP concentrations

are elevated (e.g. confluency in BHK cells), the level of cGMP will be depressed, and that rises in intracellular cGMP concentration (e.g. by addition of exogenous DBcGMP or fibroblast growth factor) will lead to a depression of cAMP levels. This model was the logical outcome from the data presented above and provided a suitable model of these results.

Unfortunately, there were many results which did not conform to this dualistic approach of cyclic nucleotide biochemistry within the cell. For example in thymic lymphocytes cAMP could stimulate division at certain concentrations (Whitfield, MacManus & Gillan, 1973b) and a similar observation has been made with chick fibroblasts (Vaheri, Ruoslahti & Hovi,1974). Polyoma-transformed BHK cells may have shown reduced cAMP levels but other tumours in vivo in the liver (Thomas, Murad, Looney & Morris,1973) adrenal (Ney, Hochella, Grahame-Smith, Dexter & Butcher,1969) and lymphatic system (Ryan & McGlurg,1973) and cAMP levels considerably higher than those in normal tissues. Furthermore, hormonal stimulation of lung cells with bradykinin was shown to cause an elevation of both nucleotides (Stoner, Manganiello & Vaughn,1973) a result which demanded the modification of the yin-yang hypothesis to allow consideration of the ratio of cGMP:cAMP as the important stimulus rather than absolute amounts. Thus any relationships found between cAMP and cell division cannot be universally applicable to all cells since cAMP had completely opposite effects in 3T3 fibroblasts, where it inhibited (Burger, et al.,1972) and thymic lymphocytes where DNA synthesis was stimulated (MacManus & Whitfield,1969a). Furthermore a hepatoma cell line (HTC) has been shown to be devoid of cAMP when in culture and to be unaffected by addition of DBcAMP (Granner, Chase, Aurbach & Tomkins,1970) so that any notion that cAMP is essential for cell division must be rejected. A cAMP-independent mutant of S49

lymphoma has also been isolated which is deficient in both cAMP binding protein and cAMP dependent histone kinases (Daniel, Litwack & Tomkins,1973b; Coffino, Bourne & Tomkins,1975a) and which was not inhibited from dividing either by DBcAMP or prostaglandin E_1 (PGE_1) which was shown to elevate intracellular cAMP by 160-fold. Also no role for cAMP in the regulation of growth of normal chick fibroblasts has been demonstrated (Hovi, Keski-Oja & Vaheri,1974; Vaheri et al., 1974). Taken together these observations indicate that cAMP cannot be universally essential for the control of proliferation and that where there is an involvement, it is as a control system superimposed upon more fundamental events which are directly involved in cell division.

The case for cGMP acting as an intracellular second messenger is complicated by the very real difficulty in measuring the compound with sufficient accuracy. Technical differences in assay procedure have led different groups to arrive at entirely different conclusions as to the importance of the nucleotide in the control of cell division. Like cAMP, cGMP passes through the cell membrane leading to losses in supernatant often discarded before the assay. The changes which occur in cGMP concentration following stimulation can be very rapid and transitory,occurring within 2 to 10 minutes of stimulation; times which are frequently not investigated. Since cGMP is present in minute quantities, attention to the purity of the sample to be assayed is essential. Generally a 2 step purification involving Dowex 50 H^+ ion exchange and QAE Sephadex A25 columns is required and serum (which can contain appreciable quantities of both cAMP and cGMP) must be ommitted from the culture medium (Coffey, Hadden & Hadden,1977). Using this procedure and radio-immuno assay a 10-50 fold increase in cGMP was detectable within 20 minutes of treatment of peripheral lymphocytes with purified PHA which was

followed 48-72 hours later by DNA synthesis, blastoid transformation and mitosis (Hadden, Hadden, Haddox & Goldberg,1972). Provided purified PHA was used there was no change in cAMP levels, suggesting it has no rôle in events leading to transformation. The rise in cGMP does not occur if the cells are stimulated with PHA or Con A in Ca^{2+} -free medium with ethyleneglycolbis-(aminoethylether) tetra--acetic acid (EGTA) to chelate the cell membrane-associated Ca^{2+}. If the cells are incubated in calcium-free medium without EGTA, a small increase in cGMP is seen following stimulation with either PHA or Con A, which is followed by a surge of cGMP production if the calcium is then replaced in the external medium, (Coffey, et al., 1977). These experiments show that the presence of extracellular calcium is required for the PHA- or Con A- stimulated rise in intracellular cGMP and that part of that calcium is EGTA-chelatable, cell-associated calcium. It should be pointed out that some workers found cAMP rose 2-3 fold during stimulation of human peripheral lymphocytes with high doses of Con A, while cGMP levels also rose two fold (Lyle & Parker,1974). At lower doses of Con A a drop in cAMP to half basal was found.

Clearly, the effects of the cyclic nucleotides vary depending on the cell type and on the concentration of nucleotide chosen. Their effects are probably dependent upon the biochemical context of the change in concentration so that either the absence or inaccessibility of the relevant protein kinase within the cell might render an elevation in cyclic nucleotide a meaningless side-effect of stimulation. Alternatively if the appropriate protein kinase had been present, the same change in cyclic nucleotide concentration would be biochemically (and perhaps functionally) significant. Having said this, it is also true that in some cells cyclic nucleotides do have a profound influence on mitogenic events but

that where involved in cell proliferation *in vivo*, it is likely that it is as one element in a co-operative sequence whose ultimate result is cell division. The sequence almost certainly contains an intimate interaction between the cyclic nucleotides and ions.

There has been increasing evidence that ions, and particularly ionic calcium (Ca^{2+}), play a central rôle in the initiation of many responses including cell division. In many cells one of the first events observed following stimulation is the uptake of many small molecules, and certain ions across the cell membrane. In peripheral lymphocytes, for example, stimulation with the plant lectin phytohaemagglutinin (PHA) elicits uptake of Ca^{2+} (Allwood, Asherton, Davey & Goodford,1971). However ionic fluxes should not be considered in isolation from other events since the influx of calcium has been shown to result in numerous changes both in ion distribution within the ionic net surrounding the cell membrane (Rasmussen & Goodman,1977) and in cyclic nucleotide concentration (Coffey, *et al.*,1977). Certainly in peripheral lymphocytes, calcium appears to be intimately involved in the initiation of DNA synthesis. When quiescent peripheral blood lymphocytes were activated by PHA (Whitney & Sutherland,1972) or thymocytes by phorbol myristate ester (Whitfield *et al.*,1973) there was an absolute requirement for extracellular calcium (Whitney & Sutherland,1972; Whitfield *et al.*, 1973). If this was removed by chelation with EGTA, no activation occurred until the calcium was replaced in the extracellular medium. Magnesium could not be substituted for calcium (Whitney & Sutherland 1972). The rise in cGMP levels induced by treatment with purified PHA (Hadden, Hadden & Golberg,1974) could not be prevented by reducing external calcium concentrations to below 10^{-4} M (Maino, Green & Crumpton,1974). However, no mitosis took place under these conditions until calcium was re-added (Maino, *et al.*,1974). These

results nominate calcium rather than cGMP as the primary mitogenic signal. It has been suggested that the initial calcium influx stimulates guanylate cyclase and therefore elevates cGMP in the cell which then further increases the permeability of the membrane to calcium i.e. a positive feed-forward reaction leading to a rapid surge of calcium into the cell (Coffey, et al.,1977) which provides the actual mitogenic signal. If external calcium concentration is less than 10^{-4} M it is probable that while sufficient membrane-bound calcium will be present to stimulate cGMP synthesis, the second surge of calcium will not be forthcoming and mitosis will not occur. Thus the importance of the interplay between ions and cyclic nucleotides becomes apparent and the result of such interplay depends not only on the relative concentrations of the ions and nucleotides but also on their temporal relationship. Thus a subsequent rise in intracellular cAMP caused by inhibition of phosphodiesterase by the rising cytosolic calcium concentration, might act as the negative feedback component, promoting calcium efflux and sequestration of calcium in mitochondrial pools. Calcium can by itself initiate DNA synthesis in thymic lymphocytes (Whitfield et al.,1969) and in higher doses in confluent cultures of Balb/c 3T3 cells though not in BHK or Balb/c SV3T3 cells (Dulbecco & Elkington,1975). Also the treatment of peripheral lymphocytes with the calcium ionophore A23187 led to blast formation provided there was calcium in the external medium (Maino, et al.,1974). It should be mentioned that in thymocytes, A23187 stimulated at two different concentration ranges one of which was dependent on external calcium, the other on external magnesium (Morgan & Perris,1976). Furthermore magnesium, like calcium, stimulated entry of quiescent thymocytes into DNA synthesis (Whitfield, Perris & Rixon,1969). While this ion has been relatively ignored in studies of the control of cell proliferation it should

not be forgotten that magnesium may play a crucial rôle in some cell activation processes and it is capable of behaving as a mitogen it its own right.

Despite the lack of convincing evidence for a universal rôle for the cyclic nucleotides in all cells, there is general agreement that in lymphoid cells cyclic nucleotides and ions act in conjunction with one another and have considerable influence on the fate of the cell. However, the precise rôle ascribed varies between authors and, at present, any model must be at least partly speculative. From a number of experiments on thymic lymphoblasts in vitro Whitfield and co-workers (Whitfield, MacManus, Rixon, et al.,1976) conclude that neither cAMP nor cGMP has any function in proliferative activation of postmitotic cells. The model proposed by this group (fig.1.5) ascribes the function of "intracycle messengers" to calcium and the cyclic nucleotides. Calcium has been shown to activate the enzyme thymidylate synthetase in broken cell preparations (Whitfield, MacManus & Gillan,1973b). The activation of this enzyme is an important prelude to DNA synthesis since it catalyses the methylation of deoxyuridine 5'-phosphate (dUMP) to deoxythymidine 5'-phosphate and it is thymine rather than uracil which is found in DNA. It is proposed that a metabolic block existing late in G_1 can be overcome by the activation of thymidylate synthetase. This can be achieved by a pulse of calcium released from the mitochondrial pool by the action of transitorily elevated intracellular cAMP observed (by this group) following stimulation of the lymphoblasts. Other observed phenomena can be fitted into this scheme. Thus it is suggested that the inhibitory action of DBcAMP on proliferation might be due to the continued presence of an analogue of a compound (cAMP) which, under natural conditions, only exerts a transitory influence in the cell. The main weakness of this model lies in the proposal that cAMP can

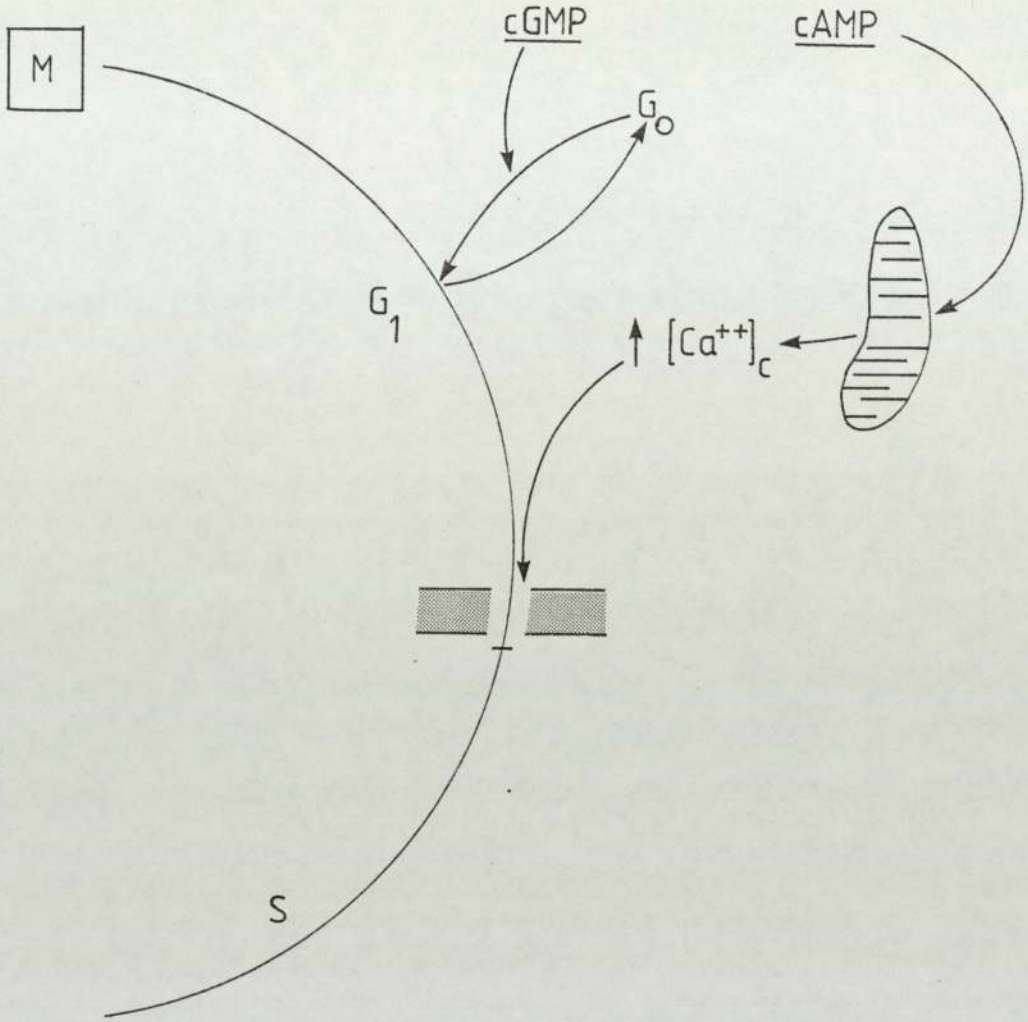


Figure 1.5 A possible site of action of cyclic nucleotides in the cell cycle. Work with thymic lymphoblasts has led one group (Whitfield et al 1976) to postulate the existence of an intracycle block late in G_1 which can be overcome in activated cells either by increasing the intracellular cAMP concentration, which causes mitochondrial calcium to be released, thus elevating cytosolic calcium ion concentration ($[Ca^{++}]_c$) or increasing the level of calcium itself. Cyclic GMP is thought to stimulate efflux of cells from a G_o state into active cycle.

stimulate calcium efflux from mitochondria. While this has been reported using isolated mitochondria from kidney (Borle,1975) the results are not reproducible (Rasmussen & Goodman,1977).

An alternative view (fig.1.6), derived from work on human peripheral lymphocytes, proposed that in these cells a membrane stimulus (e.g. with the lectin Con A) results in a calcium-dependent stimulation of guanylate cyclase causing elevated intracellular cGMP. This in turn stimulated influx of calcium resulting in elevated cytosolic calcium which stimulated further cGMP production possibly by stimulating soluble guanylate cyclase (Katagari, Terao & Osawa, 1976; Hovi, et al.,1975). A gradual increase in cAMP concentration due to phosphodiesterase inhibition by calcium might act as the negative feed-back loop, stimulating calcium efflux and thereby checking the positive feedback loop which had been set up. Although this mechanism is thought to act in smooth muscle (Rasmussen & Goodman,1977) there has been no evidence to suggest that cAMP is acting in this way in lymphocytes. It was also shown that concentrations of Con A or A23187 supraoptimal for stimulation of mitosis caused rises in cAMP. These were thought to be Mg^{2+}- rather than Ca^{2+}- dependent and were not correlated with the mitogenic activity of these substances (Coffey, et al.,1977). There are probably several causes for the different interpretations of the role of cyclic nucleotides in the control of cell proliferation. Firstly different populations of cells are used by the two groups and it is possible that the hypotheses presented reflect real differences in function of cyclic nucleotides between thymocytes and peripheral blood lymphocytes. The relative importance of cGMP in the activation of peripheral lymphocytes proposed by Hadden and co-workers almost certainly reflects the ability of this group to consistently detect mitogen-induced rises in intracellular cGMP (Coffey, et al.,1977).

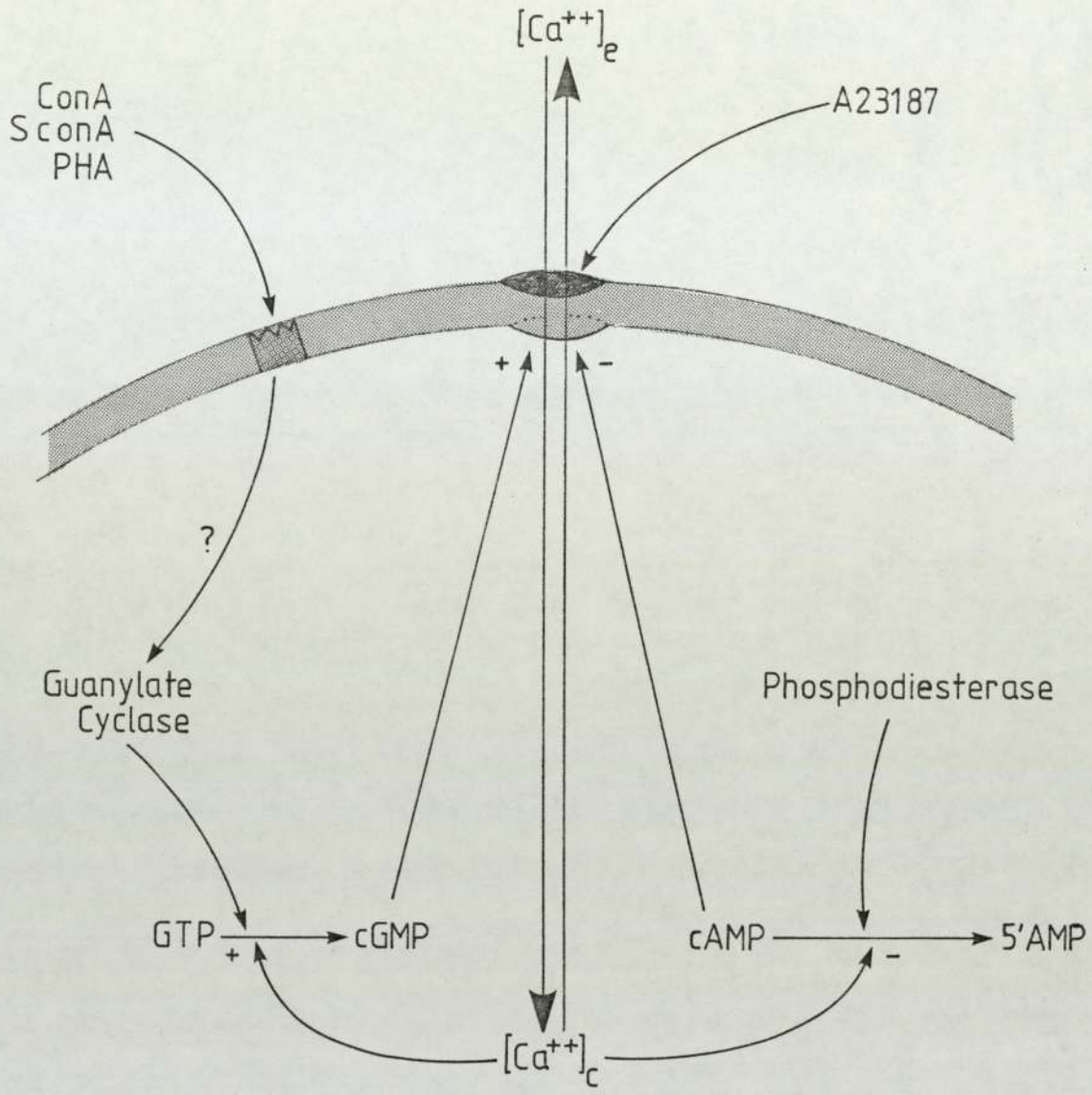


Figure 1.6 An alternative proposal for the roles of calcium and cyclic nucleotides in mitogen-stimulated peripheral lymphocytes. The interaction of mitogens such as concanavalin A (Con A) or its succinylated form (sCon A) or phytohaemagglutinin (PHA) with membrane receptors stimulates guanylate cyclase with a consequent rise in cGMP. This stimulates influx of calcium ions from the exterior ($[Ca^{++}]_e$) thus elevating the cytosolic calcium ion concentration ($[Ca^{++}]_c$). This stimulates further guanylate cyclase activity thus setting up a positive feedback. (continued over).

Figure 1.6 (continued). Ca^{2+} is known to inhibit phosphodiesterase in some cells thus giving rise to the possibility that the cAMP axis could provide a necessary negative feedback, damping the response by its actions on membrane and mitochondria. The calcium ionophore A23187 acts directly at the membrane to promote increased calcium influx down its concentration gradient. At mitogenic doses the observed rise in cGMP caused by either PHA, Con A or sCon A are calcium dependent as is the mitogenic action of A23187 (Coffey et al.,1977).

So while there is agreement that the cytosolic concentration of calcium is central to the control of cell division there is some dispute as to the way in which the ion exerts its effect. In order to appreciate more fully the biochemical effects of elevated cytosolic calcium it is necessary to consider further the intracellular control of calcium ion concentration.

In all cells the cytosolic concentration of calcium Ca^{2+}_c is subjected to complex control. The extracellular calcium concentration is approximately 10^{-3} M., whereas $[Ca^{2+}]_c$ is 10^{-6} M or less. An inward leak down this concentration gradient is countered at the plasma membrane by an outward pump. The inward flux of calcium has been commonly observed in stimulated cells and it has even been suggested that the initiation of cell division is universally accompanied by increased intracellular calcium (Berridge, 1975). There are at least two channels by which calcium can enter the cell, one potential-independent and one potential-dependent. The interaction of first messenger with membrane receptor has been shown to cause an increased permeability of a potential-dependent calcium channel which led to a depolarisation of the plasma membrane due to the rise in $[Ca^{2+}]_c$ which occurred as Ca^{2+} flowed down its concentration gradient (Rassmussen & Goodman,1977). The movement of calcium into the cell sets up a wave of disturbance in the distribution of other ions across the cell membrane. Thus increase in $[Ca^{2+}]_c$ has been shown to lead to increased potassium ion permeability (Blaustein,1974, Lake, Rasmussen & Goodman,1977; Mathews,1975; Nath & Rebhun,1973; Romero & Whittam,1971; Sarkadi, Szasz & Gardos,1976). The resulting decrease in K^+ gradient across the membrane then activates the Na^+/K^+ ATPase which leads to a fall in Na^+ in the cell. Intracellular calcium then undergoes an exchange with extracellular sodium (Blaustein,1974). Thus an

intricate intracellular ionic homeostasis exists ensuring that cytosolic calcium ion concentration is kept constant by negative feedback loops.

In addition to events at the plasma membrane, intracellular Ca^{2+} concentration is controlled by movement of calcium into and out of several intracellular pools (fig.1.7). Thus $[Ca^{2+}]_c$ depends on the binding of calcium by cytoplasmic constituents, the energy-dependent uptake of Ca^{2+} by the mitochondria and the passive leak of calcium out of the mitochondria, the deposition of a nonionic calcium--phosphate-ATP complex within the mitochondrial matrix space, the energy-dependent uptake and passive release of calcium by the smooth endoplasmic reticulum and the accumulation of calcium by secretory granules in a variety of cell types.

Cytosolic calcium concentration is therefore under the control of several factors all of which are interconnected. It is important to realize that the calcium is not evenly distributed in the cell but rather is concentrated into discrete pools, the largest of which is the calcium-phosphate-ATP complex in the mitochondria which are thus potentially a major source of cytosolic calcium. The cytosolic concentration of the calcium ion is subjected to complex intracellular control and the ion can interact with cyclic nucleotides. Few generalizations of the mode of action of calcium and the cyclic nucleotides can be made as many differences occur between cell types and in some cells no rôle for cyclic nucleotides in cell division is demonstrable. However, it is clear that in some cell types ion--nucleotide interaction may be vital physiological effector systems and that a re-arrangement of the intracellular environment (particularly ionic calcium) either leading to, or as a result of altered cyclic nucleotide metabolism is instrumental in initiating DNA synthesis. Considering the discovery that elevation of the ionic

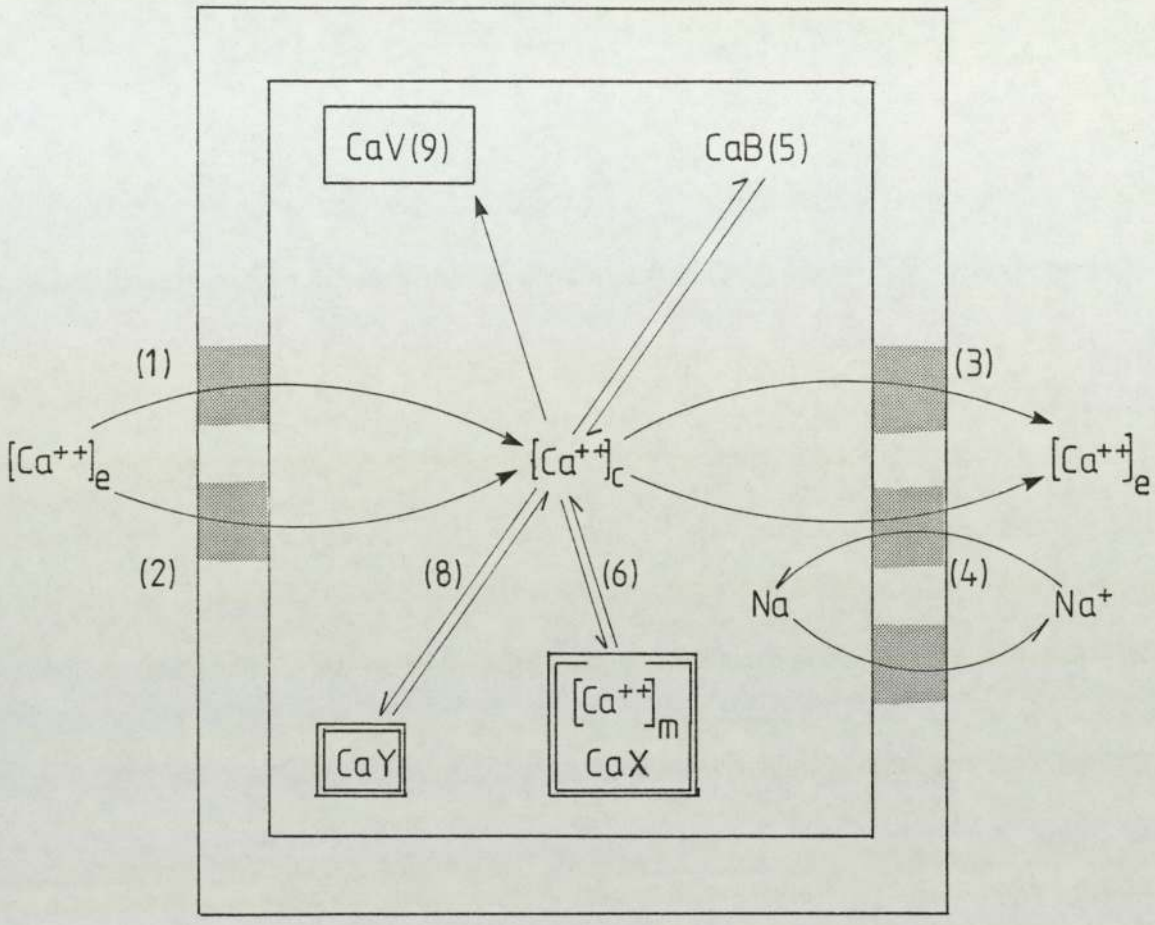


Figure 1.7 A schematic representation of the processes involved in regulation of cellular calcium metabolism in an idealized cell. Events at both plasma membrane (PM) and mitochondrial membranes (MM) are important. (1) Passive calcium (Ca) leak, unrelated to membrane potential. (2) A passive Ca leak dependent on membrane potential (3) A specific Ca pump or Ca^{2+}-activated ATP-ase. (4) A Na^{+}-Ca^{++} exchange requiring maintainence of Na^{+} gradient by the action of Na^{+}-K^{+}-ATPase. In addition, Ca^{2+} binding to cytoplasmic components (CaB) occurs (5) and there is a pump-leak system at the MM level and at the membrane of the smooth endoplasmic reticulum (8). Calcium also combines with phosphate and ATP (7) within the mitochondrial matrix space (CaX) and within the endoplasmic reticulum (CaY). Calcium is also taken up (CaV) by secretory vesicles (After Rasmussen & Goodman 1977).

calcium content of the external medium of certain cells *in vitro* could alone cause increased mitotic activity, it was of considerable interest to find an *in vivo* relationship between proliferative activity and plasma calcium concentration. Further more, it appears that the calcium homeostatic system *in vivo* may be instrumental in driving an elevated proliferative rate in some tissues in response to physiological demand. The next section describes the endocrinological control of ionic calcium in the body fluids and discusses those physiological events in which there is known to be an intimate connection between cell proliferation and calcium homeostasis.

1.4 Calcium Homeostasis and the Control of Cell Division.

In addition to the rôle which it plays in cell division, the calcium ion is involved in many other biological functions. While the skeleton represents a huge calcium reservoir, it is the relatively small pool of ionic calcium in the body fluids which is physiologically significant. Thus ionised calcium is required for muscle contraction, for enzyme activation and for the synthesis and release of acetyl choline and neurotransmitters. It is required during blood coagulation, for spindle formation in mitosis and for the production of milk. Calcium is also a component of the structure of intercellular 'cement' and is involved in the regulation of capillary permeability (Cuthbert,1970). It is not surprising to find that such a physiologically important ion is subjected to rigourous homeostatic control. The concentration of calcium in the body fluids was originally considered as one of nature's "physiological constants" (McLean & Hastings,1935) and, despite the discovery of circadian rhythms in the control of the ion (Hunt & Perris,1974; Perault-Staub, Staub & Milhaud,1974) it is still true that the plasma calcium is maintained within a very narrow concentration range. The homeostatic control of the calcium ion is achieved by the interaction of parathyroid hormone (PTH) (McLean, 1957). Calcitonin (CT) (Copp,1968) and the metabolites of vitamin D (DeLuca,1971).

Only about 1% of the body's total calcium is to be found in the body fluids, the remainder is contained in the skeleton (Catt,1971). The total extracellular calcium pool amounts to about 1 gramme in an adult human (Nordin, Marshall, Peacock & Robertson, 1975) and oscillates within $\pm$ 3% around a concentration of about 10mg. per 100ml. (10mg%) of plasma. Considering the overwhelming

excess of calcium in the skeleton and that drinking half a pint of milk adds some 340mg of calcium (McCance & Widdowson,1967), equivalent to about one third the total free calcium to the system, hypercalcaemia (elevated blood calcium) might be thought to be the major physiological derangement encountered. In fact the tendency is for a hypocalcaemia (lowered blood calcium) to develop. At 10mg% calcium, plasma is supersaturated with respect to bone (Neuman & Neuman,1958). Thus bone incubated in normal plasma or in a medium with 10mg% calcium, took up calcium whereas if incubated in calcium-free medium calcium was liberated from the bone matrix and in both cases a final equilibrium was set up at about 5-7mg% (external medium concentration). (Nordin et al., 1975). This tendency of calcium to move into the bone would, if unopposed, cause a hypocalcaemia in vivo. However, this is countered by the hypercalcaemic actions of PTH and the active metabolites of vitamin D.

Parathyroid hormone is a polypeptide secreted from the parathyroid glands. In rats there are two parathyroid glands which lie one in each lobe of the thyroid.

The synthesis of parathyroid hormone (PTH) is preceded by that of Pre-Pro PTH, an inactive precursor containing 115 amino acids. Pre-Pro PTH is converted by an N-terminal enzymatic cleavage to Pro-PTH which consists of 90 amino acids and is also inactive. Pro-PTH is subsequently converted by another N-terminal enzymatic cleavage into PTH which has 84 amino acids and a total molecular weight of 9500 Daltons (Brewer, Fairwell, Ronan, Rittel & Arnaud, 1975). The active portion of the molecule, which has been synthesized, is contained in the 1-34 region. (Brewer et al.,1975). The PTH is stored only in small quantities in the cell secretory granules before being either secreted or degraded intracellularly. The time

taken for de novo synthesis is about one hour (Habener & Kronenberg, 1978). Only small amounts of PTH are stored in secretory granules in the cells of the parathyroid glands. A hypocalcaemic episode results in the secretion of stored PTH, the cleavage of Pro-PTH into PTH and de novo synthesis of the hormone. Although hypocalcaemia is the most powerful stimulus, hypophosphataemia and hypomagnesaemia have also been shown to elicit PTH release (Buckle, Care, Cooper & Gitelman,1968; Mayer,1975) which causes a hypercalcaemia to develop (Rassmussen, Arnaud & Hawker,1964). The hormones involved in calcium homeostasis act at one or more of three sites in the body. Thus alteration of calcium flow to or from the skeleton or changes in either the rate of absorption from the gut or excretion from the kidney are factors which, when combined, provide effective control of plasma calcium within fine limits. The actions of PTH on these tissues invariably result in elevated plasma calcium. Thus PTH stimulated bone resorption (Talmage,1962), promoted calcium absorption from the gut (Rasmussen,1959) and at the kidney, PTH increased both calcium reabsorption from renal tubules (Talmage & Kraintz,1954; Kleeman,Rockney & Maxwell,1958) and renal phosphate excretion (Talmage & Kraintz,1954). The secretion rate of PTH is largely controlled by negative feedback and has been found to be inversely proportional to plasma calcium concentration in a number of species including man (Berson & Yallow,1966) pig (Arnaud,Littledike & Tsao,1970), sheep & goats (Care, Sherwood, Potts & Aurbach,1966), though in cases of extreme perturbation non-proportional responses have been found (Sherwood, Mayer, Ramberg,et al.,1968; Mayer,1975). The elevation of RNA synthesis in bone cells stimulated by PTH in vivo (Park & Talmage,1967;Owen & Bingham,1968) may represent the initiation of the synthesis of lysosomal enzymes as a prelude to osteolysis (Vaes,1968). Adenylate cyclase was also stimulated by PTH resulting in

cAMP accumulation is suspended bone cells (Chase & Aurbach,1968; Chase, Fedack & Aurbach,1969), specifically the cells of the periosteum, osteoblasts and osteocytes but not the cells of the marrow (Smith & Johnston,1975). The importance of the gut as a site of action for PTH seems minimal and probably varies between species. Thus while Rasmussen (1959) found PTH to enhance calcium absorption from the gut in rat, other workers were unable to repeat these observations (Wasserman & Comar,1961) but in both the dog (Cramer,1963) and the sheep (Care & Keynes 1964), PTH did cause some response. In the kidney PTH stimulated calcium retention and phosphate excretion (Talmage & Kraintz,1954) and whereas the action of PTH on bone was dependent on the presence of vitamin D (vide infra) the renal effects were vitamin-D-independent (Rassmussen, DeLuca, Arnaud, Hawker & Von Stedingk,1963; Arnaud, Rasmussen & Anast,1966). The kidney probably represents the single most important tissue in calcium homeostasis, particularly in short-term control, and represents the major target tissue for PTH.

Like PTH, the hormonally active metabolites of vitamin D cause responses from the target tissues which result in elevation of plasma calcium concentration. Vitamin D_3, is obtained from either dietary sources (cod liver oil is particularly rich) or biogenically from the action of ultra-violet (u/v) light on the pro-vitamin 7-dehydrocholesterol which, in vitro at least, resulted in the formation of "pre-vitamin D" which was slowly converted to vitamin D_3 (cholecalciferol) without further u/v requirement (Avioli & Haddad, 1973). Cholecalciferol is biologically inactive but has been found to undergo hydroxylation in the liver to 25-hydroxycholecalciferol (25-HCC) (Ponchon, Kennan & DeLuca,1969). This hydroxylated configuration (the major circulating type) could stimulate both bone mineral mobilisation (Raisz, Trummel & Simmons,1972) and calcium

absorption from the intestine. Then, depending on the calcium status of the animal, a further hydroxylation step took place in the kidney to either 24, 25-Dihydroxycholecalciferol, (24,25 $(OH)_2$ D_3) (Holick, Schnoes, DeLuca, et al.,1972) or 1,25--dihydroxycholecalciferol (1,25-$(OH)_2$ D_3) (Lawson, Fraser, Kodicek, et al.,1971); reactions which were catalysed by two distinct hydroxylase enzymes. (DeLuca,1974). Thus in animals with sufficient vitamin D3 but deficient in calcium 1,25-$(OH)_2$ D_3 was produced in preference to 24,25-$(OH)_2$ D_3 (Boyle, Gray & DeLuca,1971). Conversely, in animals fed with high calcium diet the 24,25-$(OH)_2$ D_3 form was produced (Omadhl & DeLuca,1973; DeLuca,1974). These responses make perfect physiological sense as 1,25-$(OH)_2$ D_3 is one hundred times more efficient at producing bone mobilisation in vitro than 24,25-$(OH)_2$ D_3 (Raisz, Trummel, Holick & DeLuca,1972) or 25-(OH) D_3 (Reynolds, Holick & DeLuca,1973a) and therefore production of the 1,25-$(OH)_2$ D_3 form is an appropriate response in conditions of calcium deprivation when hypocalcaemia is imminent. In addition to the hypocalcaemic stimulation of 1,25-$(OH)_2$ D_3 this active metabolite was produced in hypophosphataemic conditions when it stimulated bone resorption independently of PTH and promoted the uptake of phosphate from the gut (DeLuca, Tanaka & Castillo,1975). The other major site of action for 1,25-$(OH)_2$ D_3 is the gut where the steroid hormone can freely enter the cell to react with a cytoplasmic receptor protein which carries the hormone to the nucleus. Here it combined with a nuclear receptor and was found to stimulate synthesis of messenger RNA (mRNA) which in turn produced a protein called Calcium Binding Protein (CaBP) (Corradino,1973) which assists the intestinal absorption of calcium. The 1,25-$(OH)_2$ D_3 -dependent absorption of calcium from the gut is quite distinct from the 1,25-$(OH)_2$ D_3 -dependent absorption of phosphate (DeLuca, et al.,1975).

The third hormone concerned with calcium homeostasis is a linear peptide of 32 amino acid residues (M.W 3600) called Calcitonin (CT) which was found to cause a rapid, transitory hypocalcaemia when infused (Hirsch, Gauthier, & Munson,1963; Hirsch, Voekel & Munson, 1964). The hormone was originally thought to be of parathyroid origin (Copp, Davidson & Cheney,1961) but while this has been confirmed for the dog, in rat and man CT is produced in the 'C' cells of the thyroid gland (Hirsch, et al.,1963; 1964; Care,1965) which are derived embryologically from the ultimobranchial region or neural crest (Pearse,1966). The secretion of the hormone is stimulated by hypercalcaemia and a linearity between calcium concentration and CT secretion rate has been found in pigs (Care, Cooper, Duncan & Orimo, 1968; Cooper, Deftos & Potts,1971; West, O'Riordan, Copp, et al.,1973) sheep (Care,et al.,1968) rabbits (Lee, Deftos & Potts,1969) and calves (Care,Bates,Philippo, et al.,1970). However these observations apply to isolated perfused thyroid preparations only since in vivo CT is released with little or no plasma calcium change. Thus CT was secreted continuously at normal calcium levels in these animals and in rats (Klein & Talmage 1968). The main site of action of CT has been shown to be the skeleton (Munson,1971). It has a subsidiary effect on the kidney (Robinson, Martin & MacIntyre,1966) but is thought to have no effect on the gut (Cramer, Parkes & Copp,1969). Though CT inhibited PTH-stimulated bone resorption (Aliapoulious, Goldhaber & Munson,1966; Milhaud & Moukhtar,1966; Munson,1971) it also caused hypocalcaemia in parathyroidectomized (PTX) rats. Thus CT probably inhibits bone resorption independent of PTH. Some work has suggested that CT might also promote bone accretion. However, despite some support from studies of the long term effects of CT (Foster, Doyle, Bordier & Matrajt,1966) and that the simultaneous infusion of CT and calcium results in a positive calcium balance

(Pechet, Bobadilla, Carroll & Hesse,1967) the stimulation of bone accretion is not thought to be a significant action of CT.

The hypocalcaemic response induced by CT in adult rats was only 5% that of 30 day old animals (Copp,1969) presumably because of the greatly reduced bone resorption which occurred with increasing age (Copp,1969). Furthermore, adult thyroidectomy (TX) failed to produce any gross perturbations in calcium homeostasis so that much controversy has surrounded the function of CT in the adult. The hormone probably serves, in the young animal, to prevent too much bone resorption and in the adult prevents intermittent hypercalcaemic episodes which are likely to occur in pregnant and lactating females and in hyperparathyroid-induced hypercalcaemia. A variety of gastrointestinal hormones are also capable of increasing the secretion rate of CT (Cooper, Schwesinger, Mahgoub, et al.,1972; Cooper, Schwesinger, Ontjes, et al.,1972). These hormones are secreted after feeding and may provide a calcium-independent stimulus for CT secretion (Swaminathan, Bates, Bloom, et al.,1973; Philippo, Lawrence, Bruce & Donaldson,1972) the presence of which would prevent post-prandial hypercalcaemia. Although the exact rôle of CT in vivo remains to be clarified, it seems most likely that on a day to day basis the prevention of post-prandial hypercalcaemia is most significant in sheep (Philippo, et al.,1972), pigs (Swaminathan, et al.,1973) rats (Milhaud, Perault-Staub & Staub,1972) and perhaps in many other animals including man.

Although the hormones calcitonin, parathyroid hormone and the active metabolites of vitamin D are responsible for the major part of the homeostatic control of calcium there are a number of other substances which influence plasma calcium concentration. A common phenomenon in hormonal systems is the interaction of hormones and the calcium homeostatic system is no exception. Perhaps the most

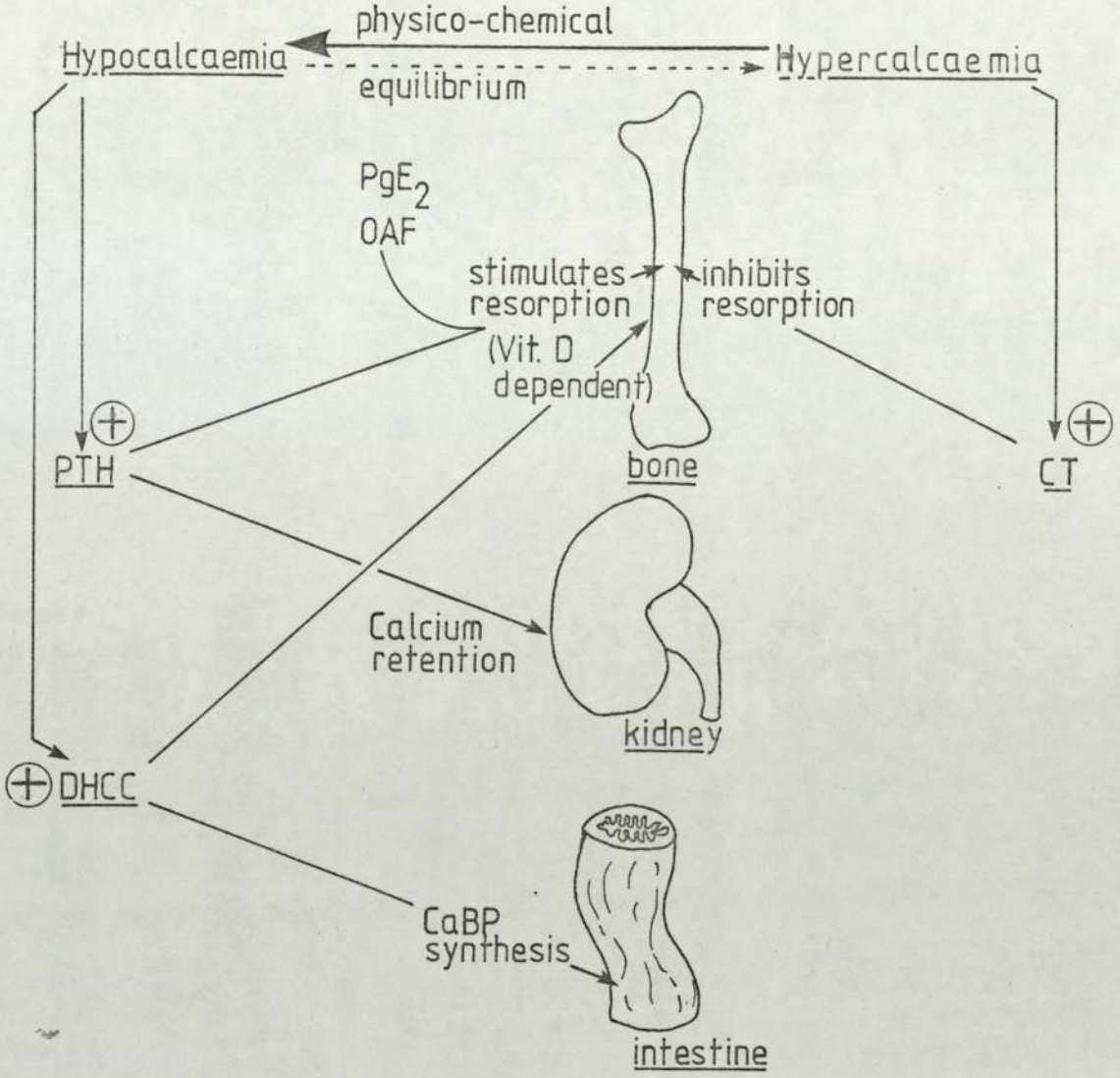


Figure 1.8. The physico-chemical equilibrium of plasma calcium tends towards hypcalcaemic conditions which stimulates parathyroid hormone (PTH) and dihydroxycholecalciferol (DHCC or Vit.D) production. Both these hormones cause a return to normocalcaemia by their actions on the tissue sites indicated. The stimulation of bone resorption by PTH is vitamin D-dependent and vitamin D also stimulates the synthesis of calcium binding protein (CaBP) which increases calcium sequestration from the intestinal lumen. During a hypercalcaemic episode, Calcitonin (CT) is secreted resulting in a return to normocalcaemia by the inhibition of bone resorption. Other agents such as Osteoclast Activating Factor (OAF) or prostaglandin E_2 (PgE_2) can also stimulate bone resorption.

important hormonal impingement on calcium homeostasis is by the sex steroid oestradiol, which inhibits bone resorption (Lafferty, Spencer & Pearson,1964; Anderson, Greenfield, Posada & Crackel, 1970) and is thus the probable cause of the variation in plasma calcium found in the oestrus cycle (Smith & Perris,1975). In addition, removal of the resorption-inhibiting oestradiol at the menopause may be one cause of progressive osteoporosis characteristic in post-menopausal women (Callagher & Nordin,1972; Gallagher, Young & Nordin,1972). Prostaglandin, particularly E_2 (PGE_2), elicited an hypercalcaemia when infused intravenously into rats (Franklin & Tashjian,1975) but not when injected intraperitoneally (Klein & Raisz,1970) which suggests that there is an efficient clearance mechanism for the hormone. Several prostaglandins are known to cause bone resorption *in vitro* (Klein & Raisz,1970; Deitrich, Goodson & Raisz,1975) but PGE_2 is most potent and has been found to be active over the range 10^{-5} - 10^{-9}M when it exhibited a linear log-dose response curve (Raisz, Trummel, Mundy & Luben,1975). Thus prostaglandins A, B and F_1 types have also been shown to stimulate bone resorption but only in the higher dose range 10^{-6} - 10^{-4}M (Raisz, *et al.*,1975). *In vivo* it is most likely that any significant effect of prostaglandin will be local, although certain tumours secrete large amounts of PGE_2 which caused significant hypercalcaemia in both mouse (Tashjian, Voekel, Goldhaber & Levine,1974a) and rabbit (Voekel, Tashjian, Franklin, Wasserman & Levine,1975). When cells in the bone marrow are stimulated to divide the greatest mitotic activity is seen immediately next to the inside of the bone tissue (Lajtha,1978) and thus adjacent to a vast supply of calcium. Leukocytes *in vitro* are known to secrete prostaglandin when stimulated with mitogen or antigen (Ferraris & DeRubertis,1974) and it is tempting to speculate that one of the results of hormonal

or antigenic stimulation of bone marrow cells *in vivo* may also be the secretion of prostaglandin. This might result in a microclimate of high calcium concentration due to enhanced bone resorption thus creating conditions which are not only favourable for heightened mitosis but which can alone drive enhanced cell proliferation.

Another immunological bone-resorbing agent is Osteoclast Activating Factor (OAF). This was first isolated from the supernatant of cultures of lymphocytes from patients with periodontal disease which had been stimulated with an antigen prepared from dental plaque (Horton, Raisz, Simmons, *et al.*,1972). Subsequently it has been found that lymphocytes stimulated with the plant lectin PHA also secrete OAF into the medium. The OAF was secreted 6 hours following PHA stimulation which is during the increased amino-acid uptake and RNA synthetic phases but before elevated DNA synthesis (Horton, Oppenheim, Mergenhagen & Raisz,1974). Like PTH, a brief exposure of OAF stimulated a prolonged resorption of rat long bone and retarded collagen synthesis. The actions of both PTH and OAF could be inhibited by calcitonin or phosphate (Raisz, *et al.*,1975). However, any possibility of OAF and PTH being similar molecules was eliminated by the lack of cross-reactivity of OAF with PTH antiserum. Likewise, OAF did not cross-react with antiserum to prostaglandins and was lipid-insoluble in contrast to prostaglandins and the metabolites of vitamin D (Luben, Mundy, Trummel & Raisz,1974). Although OAF is clearly a distinct molecule from either prostaglandin or PTH, it has been found that prostaglandin (of the E series) is required for OAF production,(Yoneda & Mundy,1979) perhaps indicating cell interaction during the synthesis of OAF. The activity of OAF has been ascribed to a single substance, destroyed by proteolytic enzymes, with a molecular weight approximately 18,000 Daltons (Lubin, *et al.*,1974). While prostaglandins and OAF are not strictly

calcium homeostatic hormones they can, under some circumstances, upset calcium homeostasis and may perform an important function *in vivo* in local promotion of bone resorption either for bone remodelling purposes or to temporarily elevate the local calcium ion concentration in the bone marrow. Extracts from a number of tissues have also been shown to cause hypocalcaemia *in vivo* notably a large molecular weight fraction from calf thymus (Mizutani,1973) but as they have no defined physiological function they will not be discussed further here.

It was first realized that the calcium homeostatic system affected cell proliferation rates *in vivo* when heightened mitotic activity was found in the thymus (Perris & Whitfield,1967a) and bone marrow (Perris, Whitfield & Rixon,1967; Perris & Whitfield, 1967b, of rats injected with calcium chloride. Parathyroid extract had essentially the same effect probably mediated by the induced hypercalcaemia. Extirpation of the parathyroids, which leads to severe hypocalcaemia (Hirsch, Gauthier & Munson,1963) resulted in reduced mitotic activity in the bone marrow (Rixon 1968; Perris & Whitfield,1971; Rixon & Whitfield,1972) which was accompanied by a progressive hypoplasia (Rixon & Whitfield,1972). The reduction in bone marrow cellularity was due to depletion of lymphoid and erythroid elements while the myeloid series was unaffected. In addition, the thymus became severely atrophic following parathyroidectomy (PTX). The possibility of this atrophy being caused by post-operative elevation of corticosteroids, which are known to have lympholytic properties, has been excluded (Perris, Weiss & Whitfield,1970). Furthermore the mitotic activity of the bone marrow and thymus could be partially or wholly restored by administration of calcium salts either orally or intraperitoneally or by injections of PTH (Perris *et al.*,1970; Perris & Whitfield,1971). It seems most likely from

these results that the plasma calcium concentration rather than PTH itself is responsible for the elevation or depression of mitotic activity in bone marrow and thymus. A good correlation between mitotic activity in bone marrow or thymus and plasma calcium concentration was obtained over the range of values achieved by manipulations of the homeostatic mechanism (Rixon,1968).

Excellent support for the idea that mitotic activity is directly proportional to calcium concentration has come from *in vitro* studies of thymocytes. Thus raising the concentration of calcium in the medium from 1.0 to 1.2 mM increased the DNA synthesis of thymocytes (Whitfield, MacManus, Youdale & Franks,1971) and the number of cells arriving at mitosis over a six hour incubation period (Perris,*et al.*, 1967; 1968) while lowering the extracellular calcium below 0.2 mM greatly reduced DNA synthesis and proliferative activity. Bone marrow cells *in vitro* also respond to stimulation with elevated extracellular calcium by initiating a very rapid burst of proliferation and an increase in the number of reticulocytes formed (Morton,1968). This directly parallels the *in vivo* observation that injections of calcium chloride enhanced reticulocyte formation in the bone marrow and increased the number of these cells in circulation (Perris & Whitfield,1971). Furthermore, many mitogenic agents have an obligatory requirement for the presence of calcium. In addition, the mitogenic actions of PTH (Whitfield, Rixon, Perris & Youdale,1969; Whitfield, MacManus, Youdale & Franks,1971) detergents, agmatine, poly L-lysine (Whitfield, Perris & Youdale, 1968) growth hormone, prolactin, neurohormones (Whitfield, *et al.*, 1969; Whitfield, Perris & Youdale,1969) cortisol (Whitfield, MacManus & Rixon,1970) acetyl choline, histamine and insulin (Morgan, Hall & Perris,1976) on thymocytes are ablated in calcium-free medium.

It has now been established that a number of physiological situations exist in which there is a calcium-dependent stimulation of bone marrow mitotic activity. In each case the elevated mitotic activity is associated with a hypercalcaemia and neither elevated plasma calcium nor enhanced proliferation is found in aparathyroid animals. Thus if erythropoiesis was stimulated by haemorrhage (Perris, MacManus, Whitfield & Weiss,1971), pregnancy (Perris,1971) injections of cobaltous chloride (Perris,1971) or erythopoietin (Hunt & Perris,1973; 1974a) a parathyroid-dependent rise in bone marrow mitosis was seen. Plasma calcium concentrations are also known to vary in a circadian rhythm and during the oestrus cycle and in both these cases the calcium fluctuations were directly paralleled by bone marrow mitotic activity (Hunt & Perris,1974b; Smith & Perris, 1976). In the young animal a high rate of proliferation is found in many tissues which is associated with a high plasma calcium. As the proliferative activity declines with increasing age, so the plasma calcium level falls (Perris, Whitfield & Tölg,1968).

In summary elevation or reduction of calcium concentrations of culture medium results in an increase or decrease of mitotic activity of the cells and there is an absolute requirement for calcium for the mitogenic action of hormones and many other agents. If the concentration of plasma calcium (in vivo) is artifically altered, there is a similar parallel between the concentration of calcium and mitotic activity of bone marrow or thymus. Perhaps most importantly in a number of physiological situations characterized by elevated bone marrow proliferation, a hypercalcaemia invariably accompanies the increased mitotic activity. From these studies came a number of disparate observations which formed the basis of this present study. During the immune response there is a physiological demand for rapid proliferation of lymphoid cells and it has been

shown that while injections of calcium chloride enhanced the antibody response of mice, calcitonin caused retardation (Braun, Ishizuka & Seeman,1970). Furthermore, before the significance of the influence of the calcium homeostatic system on cell proliferation had been realized, it was shown that injection of calcium chloride enhanced the survival time of irradiated rats (Rixon & Whitfield, 1963) a phenomena which may be due to enhanced immunity. The precedent had been set, therefore, for the possibility that the calcium homeostatic system may be involved in the lymphoproliferative events of the immune response. The major purpose of the present study was to test the hypothesis that the calcium homeostatic system is involved in the control of proliferation of lymphocytes in the immune response.

Before considering the immune system and immune products which are specific regulators of lymphoid proliferation, it is necessary to briefly consider the humoral factors which participate in the general control of cell division in vivo.

1.5 Hormones, Chalones and Cell Proliferation.

The cells of the body are exposed to a wide variety of humoral factors which can modify their rate of proliferation. Many of these factors are hormones which exhibit mitogenic properties as only one of a range of physiological effects. Thus the continued presence of testosterone has been shown to be required by the prostate, coagulating and preputial glands of the male which become hypoplastic in castrated mice (Allison, Appleton, McHanwell & Wright,1976). The uterus of mammals shows cyclic variation in size caused by the proliferation and differentiation of the endometrium. While the control mechanisms are complex these responses are in part due to the proliferative response of the endometrial cells to oestradiol and if implantation should occur then interaction of several hormones results in further proliferation and differentiation. It has been shown that both thyroxine and oestrogens can stimulate the cells of the epidermis (Ebling,1974) and that the intestinal mucosa can be stimulated by pentagastrin (Pansu, Berard, Dechelette & Lambert,1974) and vitamin D (Speilvogel, Farley & Norman,1972). A wide variety of hormones have been shown to be mitogenic *in vivo* though the mechanism of action varies. Thus while insulin may act with membranal receptors, oestradiol probably exerts its effect via cytoplasmic receptors. PTH on the other hand, is probably mitogenic only because it causes hypercalcaemia, which is in itself sufficient mitogenic stimulus. It must be remembered that the demonstration of mitogenic properties in a hormone does not mean that this is a major action of the hormone (though it may be) nor does it necessarily suggest any physiological significance.

It is important to treat with caution results obtained from cells *in vitro* which may respond differently because they are

deprived of their normal humoral environment. Bearing this in mind, the thymic lymphocyte has proved particularly susceptible to mitogenic stimulation by a large number of hormones (table 1). Furthermore some of the agents which stimulate thymic lymphoblasts such as bradykinin, histamine and prostaglandin E_1 are known to have a rôle *in vivo* thus increasing the possibility that these studies may well have physiological significance.

In 1913, seven years after Starling proposed the term "hormone" for a stimulatory signal substance, the term "chalone" was suggested for those substances which were inhibitory in action (Schäfer,1913). Although the proposal was not accepted and the term "hormone" today signifies either a stimulatory or inhibitory substance, the word "chalone" was resurrected by Bullough (1962) to describe the type of tissue-specific, anti-proliferative substances which were being extracted from pig epidermis. These substances were obtained by aqueous extraction using apparatus cooled to $4^{\circ}C$ which provided a preparation which could be further purified by molecular sieve, dialysis or alcohol fractionation. Chalones have since been found in many other tissues (table 2) and the following properties are now generally accepted as essential features of chalone activity.

1). Chalones are produced in and present in the tissues on which they selectively act.

2). Chalones may act locally by diffusion in the tissue, or generally through the circulation.

3). Chalones are water-soluble molecules.

4). Chalones are tissue specific, but species non-specific.

5). Chalones act mainly in the late G_1 phase by delaying entry of cells into DNAsynthesis and/or in the late G_2 phase retarding entry into mitosis.

6). Chalone action is reversible and chalones do not injure cells or cell membranes.

Table 1: The stimulatory action of Hormones on *in vitro* thymocyte proliferation.

Hormone	Reference
Cortisol	Whitfield, MacManus & Rixon, (1970).
Bradykinin	Perris & Whitfield, (1969).
Adrenalin	MacManus, Whitfield & Youdale, (1971).
Growth Hormone	Whitfield, *et al.*, (1969)
Oxytocin	" " "
Prolactin	" " "
Parathyroid Hormone	" " "
Prostaglandin E_1	Whitfield, MacMannus, Braceland & Gillan,(1972).
Vasopressin	Whitfield, MacManus, & Gillan (1970).
Calcitonin	Whitfield, MacManus, Franks, Braceland & Gillan, (1972)
Histamine	Morgan, Hall & Perris, (1976).
Glucagon	" " " "
Insulin	" " " "

Table 2: The wide variety of tissues in which a Chalone System has been reported.

Cell- or tissue-type	Reference.
Lymphocytes	Moorhead et al (1968) Attallah & Houck (1976)
Granulocytes	Rytömaa (1976) Paukovits (1976)
Erythrocytes	Kivilaakso & Rytömaa (1971)
Melanocyte	Bullough & Lawrence (1968)
Fibroblast	Houck, Sharma & Cheng (1973)
Epidermis	Bullough & Lawrence (1964) Elgjo (1976)
Sebaceous Gland	Bullough & Lawrence (1970)
Kidney	Saetren (1963)
Liver	Verly (1976)
Lung	Simnett, Fisher & Heppleston (1969)
Intestinal Epithelia	Tutten (1973)
Uterus	Chevalier & Verly (1975)
Testis	Cleremont & Mauger (1974)
Muscle	Florentin, Nam & Janakidevi et al.,(1973)

7). Chalone action is short-lived, which may be due to chalone antagonists, sometimes called anti-chalones.

The mechanism of action of chalones is not known. Possibly in conjunction with other hormones they may influence cyclic nucleotide metabolism (Atallah & Houck,1976). This proposal is purely speculative however and it is not even known whether the site of action is membranal or cytoplasmic. The concept of an anti--proliferative substance may help to explain many phenomena such as liver regeneration (Verly, Deschamps, Pushpathadam & Desrosiers, 1971; Verly,1976)and wound healing (Bullough & Lawrence 1960; Iversen, Bhangoo & Hansen,1974). Removal or destruction of tissue would lead to the sudden withdrawl of the chalone thus inducing a lack of suppression and a sudden wave of proliferative activity in the remaining cells adjacent to the wound. The potential use of such substances in retarding tumour growth or in synchronizing the cells of such a tissue prior to radiation therapy is an exciting possibility.

Chalones have been extracted from a wide variety of tissues including lymphoid organs and lymphocyte chalone has been shown to inhibit a number of physiological and immunological responses both in vitro and in vivo. Thus pig lymph node extract resulted in inhibition of transformation of human peripheral blood lymphocytes (Moorhead, Paraskova-Tchernozenska, Pirrie & Hayes,1969; Jones, Paraskova-Tchernozenska & Moorhead,1970). A similar chalone has been extracted from human lymphocytes, bovine spleen (Lasalvia, Garcia-Giralt & Macieira-Coelho,1970) and thymus (Kiger,1971) and from lymphoid organs of rat, dog, rabbit and guinea pig (Houck & Irausquin,1973; Attallah, Sunshine, Hunt & Houck,1975). The chalone has been variously reported to have a molecular weight of between 30,000-50,000 Daltons (Garcia-Giralt, Lasalvia, Florentin

& Mathe,1970; Garcia-Giralt, Morales, Lasalvia & Mathe,1972) or 50,000-70,000 Daltons (Moorhead, et al.,1969). Further work has indicated that the biologically active component may be a peptide (10,000 D) complexed to an inert, low molecular weight RNA carrier by strong ionic bonds (Attallah & Houck,1976).

The exact specificity of the lymphocyte chalone has not yet been determined. There is some evidence to support the idea that chalone derived from T-cells will specifically inhibit T-cell function. In contrast chalone from B-cell seem to inhibit both B- and T-cell function (Attallah & Houck,1976). The results are not conclusive however since putative T-cell extract from the thymus was found to inhibit transformation induced by B-lipopolysaccharide from Salmonella typhosa (Attallah,et al.,1975), a substance which is known to stimulate B-cells (Peavy, Adler & Smith,1970).

The action of lymphocyte chalone in vivo is invariably immunosupressive. Thus lymphocyte chalone inhibited neonatal thymic growth (Chung & Hufnagel,1973) reduced the number of antibody forming cells (Garcia-Giralt et al.,1970; Florentin, Kiger & Mathe 1973) and increased the survival time of mouse skin allografts (Kiger, Florentin, Garcia-Giralt & Mathe,1972; Houck, Attallah & Lilly,1973) and xenografts (rabbit to mouse) (Chung & Hufnagel,1973).

Injection of sub-lethally irradiated F_1 animals with parental strain lymphocytes leads to a progressive wasting of the recipient's body tissues and eventual death. This is caused by the failure of the F_1 lymphocytes to recognize parental strain lymphocytes as foreign. The donated lymphocytes, which recognize F_1 tissue as foreign, are thus able to mount an immune response against F_1 tissues which proceeds unchecked. This phenomena is called a graft-versus--host reaction (GVHR). Pretreatment of the parental cells with

chalone was found to enhance the survival time of F_1 recipients (Garcia-Giralt et al.,1970) a result which has been confirmed independently (Kiger et al.,1972; Kiger, Florentin & Mathe,1973a). The distinct possibility that these results were obtained by cytotoxic effects of the chalone on the parental lymphocytes was satisfactorily eliminated (Garcia-Giralt, Rella, Morales, et al.,1973; Kiger, Florentin & Mathe,1973b).

Although the concept of a mitotic inhibitor present in tissues is very attractive, the evidence for the existence of chalones is still not totally convincing. Some earlier work, particularly with epidermal chalone suffered from the presence of bacterial contaminants, extracts of which mimicked the putative chalone activity (Mohr, Boldingh & Althoff,1972; Mohr, Boldingh, Emminger & Behagel, 1972). However, the research is beginning to look more promising and control experiments more stringent but it is surprising to find that the relatively crude preparations of lymphoid chalone, while undoubtedly immunosuppressive, are not more cytotoxic. The assays for chalone activity invariably involve measuring some function of cell division and therefore it is of prime importance to control for the possibility of non-specific cytotoxicity, for as Houck has pointed out "dead cells do not divide and dying cells divide damn slowly!" (Houck & Hunt,1976).

Cells within the tissues of an intact animal are thus exposed to a wide variety of humoral factors, both systemic and local, which can influence whether the cell is pushed into, or retarded from, more rapid proliferation. The effect of these factors at the cellular level remains to be completely elucidated but it seems possible that any proliferative effect may be mediated by alterations in cyclic nucleotide metabolism and/or ion distribution. The calcium ion appears to play a central rôle in proliferative responses. All

of these factors equally apply to those cells of the immune system which are capable of dividing. The remainder of this thesis will consider lymphoid cells almost exclusively with a view to obtaining a clear picture of the additional mechanisms by which controlled proliferation is achieved in the immune system, and the relevance of this to the effective eradication of foreign substances.

1.6 The Immune System.

In its broadest sense the term immunity applies to all the mechanisms whereby animals are protected from infection. These include physical barriers, non-specific humoral factors and effector cells, as well as specific immune components. The non-specific mechanisms constitute the first line of defence and are largely responsible for the natural (innate) immunity which animals possess to the vast majority of environmental micro-organisms. The specific mechanism deals primarily with those organisms which are capable of passing the non-specific barriers.

Specific immunity is always acquired. Its cardinal features are the ability to recognize and memorize foreign substances (antigens) thus leading to enhanced capacity to resist infections. It is not an absolute phenomenon, however, and the outcome of an infection depends not only on the type of infectious agent but also on the amount, its virulence and the mode of entry.

There are two main types of immune response, antibody-mediated or humoral immunity and cell-mediated immunity. The humoral immune response is characterized by the production of blood-borne antibody molecules which can specifically bind to the foreign substance or antigen which initiated the reaction. Antigens such as heterologous serum protein or bacterial lipopolysaccharides are potent simulators of humoral immunity. Cell mediated immunity (CMI) on the other hand, is more local in expression and the reaction is delayed. The secreted products of CMI reactions (lymphokines) are short lived and generally confined to the immediate area of the immune reaction. CMI reactions are mounted in response to such antigens as non-isogeneic skin grafts (i.e. from a different strain or species) and tumour cells. The lymphocyte plays a central rôle in both types of immune response and

different populations of these cells are responsible for the two types of immunity.

In the late 1950's the lymphocyte was still being referred to as "a short lived cell of no known function" (Yoffey & Courtice,1956), though soon after the importance of lymphocytes to the immune response was demonstrated (Gowans & McGregor,1965). It was not long before it was realized that while lymphocytes appeared to be homogeneous population of cells by standard histological techniques, they exhibited a clear functional heterogeneity. Work on the domestic fowl showed that neonatal removal of the bursa of Fabricius, a cloacal lymphoepithelial organ characteristic of birds resulted in an inability to synthesize antibody while the capacity to reject skin allografts was retained (Muller, Wolfe & Meyer,1960; Warner & Szenberg,1964; Perey, Cooper & Good,1968, Sutherland, Archer & Good,1964).

In contrast neonatal thymectomy(NTx) or adult thymectomy (ATx) and irradiation caused a drop in the number of peripheral and thoracic duct lymphocytes. These procedures also led to the depletion of particular areas of the secondary lymphoid tissue such as the periarteriolar lymphatic sheath of the spleen (Miller & Osoba,1967). Several aspects of the immune responses were also impaired. Thus NTx animals were unable to reject skin allografts and failed to show delayed hypersensitivity. Antibody responses to some but not all antigens were also reduced. These results which were complimented by clinical observations on patients with immune deficiency diseases (Good, Biggars & Park,1971) and by studies on the nude mouse (nu/nu) which is congenitally athymic led to the idea that lymphocytes could be classified into two distinct functional types. The bursa-derived lymphocytes (B-cells) are the precursor cells of the plasma cell which secretes antibody. On the other hand, the thymus and the

T-cells derived from it are necessary for the proper development of cell-mediated immunity. Thus B-cells develop into cells which produce humoral antibody and are thus required for humoral immunity to antigen while T-cells are required for the expression of cell--mediated immunity.

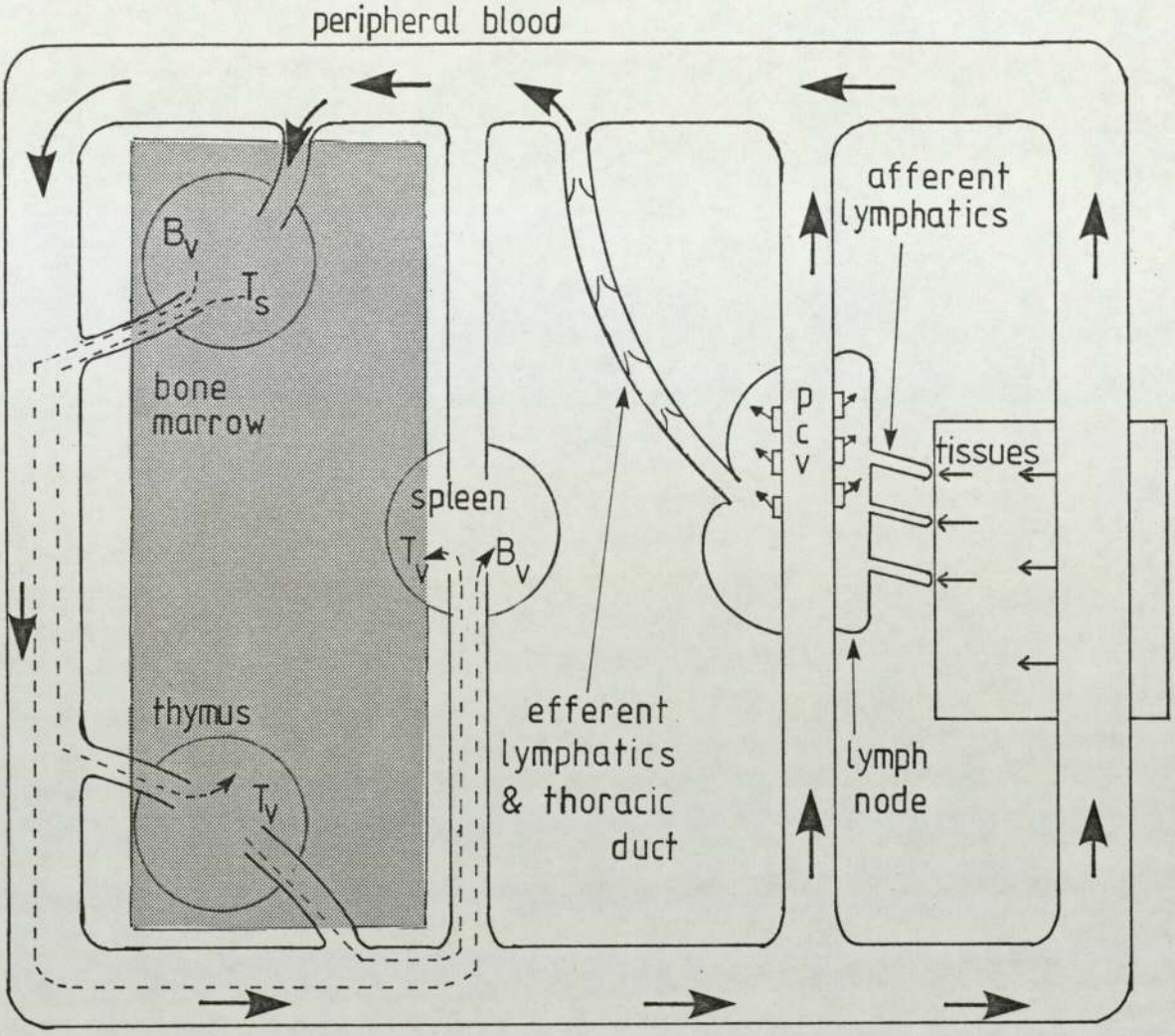
Lymphocytes are not isolated particles but form part of the "Lymphon", a collective term to describe the lymphoid tissues and their constituent cells. Both B- and T-cells are derived from pluripotential stem cells found in the yolk sac of the embryo and later in feotal liver and spleen (Owen,1977). In birds, the precursor B-cell differentiates in the bursa of Fabricius which remains the site of B-cell production throughout the life of the bird. In mammals the site of B-cell maturation is probably multifocal despite the suggestions that gut-associated lymphoid tissue (GALT) such as the appendix (Archer, Sutherland & Good,1963), tonsils (Peterson, Cooper & Good,1965), Peyer's patches (Perey, Cooper & Good,1968) and foetal liver (Owen, Cooper & Raff,1974) may act as bursa-equivalents. Although there is no 'central' lymphoid organ exactly analagous to the avian bursa, antibody forming cells in mammals are probably derived from the bone marrow (Davies, Leuchars, Wallis et al.,1967). Unlike the bursa of Fabricius, however, the bone marrow is not essential for B-cell maturation (Kincade, Moore, Schlegel & Pye,1965; Phillips & Miller,1974).

The maturation of T-cells requires the specific influence of the thymic environment. Following development of the thymus from pharangeal pouch III (Rogers,1929) it becomes populated with blood--borne stem cells from the bone marrow. These proliferate and differentiate into mature T-cells which leave the thymus and populate peripheral lymphoid tissues.

The thymus and the bursa (or it equivalent(s)) are called primary lymphoid organs. It is characteristic of the cells of these organs to exhibit a high rate of proliferation. In addition the cells are usually marked by an independence from antigenic stimulation, particularly amongst the more primitive cells which presumably do not possess the relevant membrane receptors. As a corollary to this, neither the thymus nor the bone marrow is a major source of antibody despite being large lymphoid organs (Le Douarain, 1977).

After maturation in the primary lymphoid organs, the lymphocytes become disseminated to the secondary lymphoid organs. These include the lymph nodes, spleen and gut-associated lymphoid tissues. These secondary lymphoid tissues are greatly reduced in size in animals reared under gnotobiotic (germ-free) conditions which suggests they may be important in the response to infectious agents. Indeed although lymphopoietic activity is normally low, germinal centres within secondary lymphoid tissues exhibit a proliferative response to antigens.

Following emigration from primary lymphoid tissues the majority of naive lymphocytes home to anatomically discrete areas in secondary lymphoid tissue where they remain predominantly sessile (Stober,1972). The anatomical arrangement in the tissue may well serve to 'present' the antigen in a form which provides optimum potency during the first encounter with a novel antigen. Some do however circulate (fig.1.9) but it seems that lymphocytes which have 'experience' of a previous encounter with a given antigen i.e. memory or primed lymphocytes, are the predominant form which circulate through the thoracic duct, blood and lymph nodes (Sprent,1975). The circulation of memory cells provides these cells with maximum opportunity of re-encountering the antigen to which they are primed. In contrast sequestration of the



Key.

- → Recirculating lymphocyte pool
- B_v Virgin B-cell
- T_v Virgin T-cell
- T_s Lymphoid stem cell (putative T-cell precursor)
- PCV Post capillary venule
- ▒ Not part of recirculating lymphocyte pool.

Figure 1.9 The major lymphocyte traffic patterns in the mature lymphon. T_s represents the migration of stem cell ((?) precursor T-cells) from the bone marrow to the thymus. Virgin B & T cells (B_v and T_v) tend to migrate preferentially to the spleen though this is not the only site where they may be found. Following antigenic stimulation B and T cells show an increased tendency to become circulating lymphocytes. Lymphocytes which enter the spleen by the circulation can leave via the splenic vein. In lymph nodes, however, cells entering through the post capillary venule (PCV)or afferent lymphatics can only leave in the efferent lymphatics which join with the thoracic duct which drains into the main circulation once more.

virgin lymphocytes probably reflects the importance of the spleen as a site for the presentation of antigen in a highly immunogenic form.

Cells containing immunoglobulin (Ig) but not yet bearing it on their surface are regarded as pre-B-cells and exist in foetal liver up to birth (Raff, Megson, Owen & Cooper,1976). T-cells arise from the same stem cells as B-cells, and appear to undergo some differentiation *in situ* prior to migration to the thymic environment. Thus while cells exhibiting T-cell markers have been demonstrated in athymic 'nude' mice (Roelants, Loor, von Boehmer, *et al.*,1975) these probably represent thymic precursor cells unable to differentiate further in the absence of a thymus and which are presumably immunologically non-functional. The pre-T-cells migrate to the foetal thymus where they develop the theta (θ) antigen (a surface characteristic of T-cells to which antiserum can be raised) as early as the fifteenth day of gestation (Owen & Raff,1970). However, full immunological maturation as measured by the ability of the cells to react, by proliferation *in vitro*, to the presence of allogeneic (different strain) lymphocytes (a Mixed Lymphocyte Reaction) is first present at or just before birth (Mosier,1974). Thus there is considerable delay between initial maturation and functional differentiation. It is interesting to find that agents which elevate endogenous cAMP can induce the expression of T surface antigens on cells of foetal liver *in vitro* (Scheid, Hoffmann Komuro *et al.*,1973). Furthermore at least one substance Thymic Humoral Factor (THF) isolated from the thymus (Kook, Yakir & Trainin,1975; Trainin, Small, Zipori *et al.*,1975) does stimulate adenylate cyclase and result in elevation of endogenous cAMP (Kook & Trainin,1974,1975). These findings provide evidence for the suggestion that the differentiation of lymphocytes within the thymic environment is mediated by humoral factors.

The recognition that two functionally distinct populations of lymphocytes existed was a vital step towards further elucidation of the immune system. When procedures for the separation of T- from B-lymphocytes were refined, closer studies were possible. It was found that the surface architecture of the two populations was subtly different. The characteristic units of the cell membranes, unique to either T- or B-cells are called "surface markers". One such marker the θ antigen mentioned earlier is found on mature mouse T-lymphocytes and is shared with brain tissues. If brain cells are broken and the membrane fragments used to raise antibody in (say) a rabbit then the resulting antiserum will contain antibodies which not only bind to mouse brain cells but also to mouse T-cells. By conjugating such antibodies to fluorescent dye T-cells can be picked out from a mixed population because they will appear to glow under u/v light.

Once immunologists became used to the idea of sub-populations of lymphocytes, it became easier to consider the possibility of further sub-sets. Evidence has been presented which suggests the possibility of two subpopulations of T-cells in the mouse T_1 which are essentially spleen seeking, relatively rich in θ and insensitive to anti-lymphocyte serum, and T_2 which migrate preferentially to the lymph nodes, have little surface θ antigen and are comparatively sensitive <u>in vitro</u> to anti-lymphocyte serum (Raff & Cantor, 1971) T_1 cells are short lived and disappear quite rapidly after adult thymectomy, which is perhaps indicative of function for the thymus in the adult despite considerable involution. Further work on the heterogeneity of T-cells let to the idea that there were in fact several sub-populations of T-cells. Thus helper T-cells (T_H), killer T-cells (T_K) cytotoxic T-cells (T_C) supressor T-cells (T_S) and embryonic T-cells (T_E) have all been characterized. The discovery that these functional sub-sets showed different surface structure in

terms of an allelic antigen, Ly, allowed a clearer picture to be obtained. Thus just as Thy_1 has become the antigenic marker for T-cells, so Ly is becoming the marker for sub-populations of T-cells. It now seems likely that the differentiation of the presumed T-stem cell into the precursors of the various T-subsets is antigen independent i.e. that the precursor cells are already committed to the type of T-cell which they will become before they encounter antigen (Cantor & Boyse,1975).

B-cells show surface bound immunoglobulin in all species studied but on the whole, lack useful antigenic markers of the type found in T-cells. Studies suggest that IgM is the first immunoglobulin to appear (Kincade & Cooper,1971) but that there is later a switch to either IgG or IgA (Lawson, Asovsky, Hylton & Cooper,1972; Pierce, Solliday & Asovsky,1972). The IgM-bearing cells are probably uncommitted cells which differentiate to bear the other Ig class following exposure to antigen (Wigzel,1970; Greaves & Hogg,1971). Surface IgD can also act as a B-cell receptor for antigen and it is possible that IgD^+ cells are memory B-cells (Parkhouse & Abney,1975). An alternative view is that antigen interaction with an IgM^+ cell in the absence of T-cell help results in tolerance while interaction with an IgM^+ IgD^+ cell with T-cell help results in IgM production and IgM memory. Other cells bearing either IgM^+ IgG^+ IgD^+ or IgM^+ IgA^+ IgD^+ develop into secreting or memory cells for IgG or IgA respectively on antigen contact with T-cell help (Vitetta & Uhr,1975).

Further B-cell heterogeneity is demonstrated by the response of B-cells to known B-cell mitogens such as pokeweed mitogen (PWM) or bacterial lipopolysaccharide (LPS). Even under optimal conditions only 50% of B-cells respond to either mitogen (Greaves & Janossy,1975). The full response to LPS or PWM would be a high rate of antibody synthesis following a rapid proliferative phase. Some cells

proliferate without antibody production while others fail to respond at all. These results are probably indicative of the ontogeneic sequence in the adult. "Early" types may require other potentiating factors (see section 1.7) while mature types do not. This suggestion is supported by the observation that the thymus-dependent antibody responses (those in which B-cells produce antibody only if there is help from T-cells) arise earlier in ontogeny than the thymus--independent ones (where no T-cell help is required) (Andersson & Blomgren,1975).

Some lymphocytes show neither surface immunoglobulin nor T-cell surface markers and are thus designated "Null cells". This is almost certainly a heterogeneous population. It is probably from the null cell population that K-cells are derived. These cells bear F_c receptors and are capable of killing antibody-coated target cells (Greenberg, Hudson, Shen & Roitt,1973).

It should be remembered that lymphocytes only form part of the immune system and that phagocytes also play an important part in the elimination of antigen. While phagocytes are capable of engulfing and neutralizing antigen, they do not possess any specificity though this can be conferred onto macrophages (macro-phagocytes) passively by lymphocytes. Finally, T-cells, B-cells and macrophages co-operate in the effective expulsion of antigen (section 1.7).

Only a very limited number of virgin B-lymphocytes will be able to bind any particular antigen. This is because each cell will have surface Ig which is specific for different molecular features or antigenic determinents sometimes called epitopes. Clearly a large antigen such as a foreign erythrocyte will have many different epitopes available on its surface. Consequently B-cells with different surface Ig specificities, each corresponding to an epitope on the antigen, will be able to respond. Even so, very few cells in

a population are able to bind to a novel antigen and far too few to produce appreciable amounts of antibody. Under the correct conditions the binding of antigen to the B-cell elicits a mitogenic response (section 1.8). The result is a rapid proliferation of the activated lymphocytes so that after 4-5 days, and 6-10 rounds of division later, there exists a huge clone of plasma cells which are descended from the original activated cells (fig.1.10). The antibody secreted by these plasma cells is of the same epitope specificity as that which was surface bound to the virgin B-cell. It follows that the secreted antibody binds specifically to the epitope which first activated the B-cell.

This hypothesis forms the basis of the "clonal selection theory" of immunity (Burnet,1959) and is really an example of a cell recognition mechanism combined with a biological amplification system. Recognition and amplification are at the heart of immune responses.

The antibody secreted by plasma cells has been shown to belong to the γ-globulins and the term "immunoglobulin" was introduced to describe proteins with known antibody activity (Heremans,1960). The term immunoglobulin is a functional rather than a physical description. There are five known classes if immunoglobulin which, because of the nature of their common activity form a family of proteins. The classes are designated immunoglobulin G (IgG), IgM, IgA, IgD and IgE which are placed here in descending order of normal serum concentration.

The IgG molecule (fig.1.11) consists of four polypeptide chains bound together by sulphydryl bonds to form a shape which can be represented schematically by a 'Y'. Cleavage of the molecule by papain leads to a crystalizable fragments (Fc) and a fragment which will bind to antigen (Fab). The antigen binding site is found at

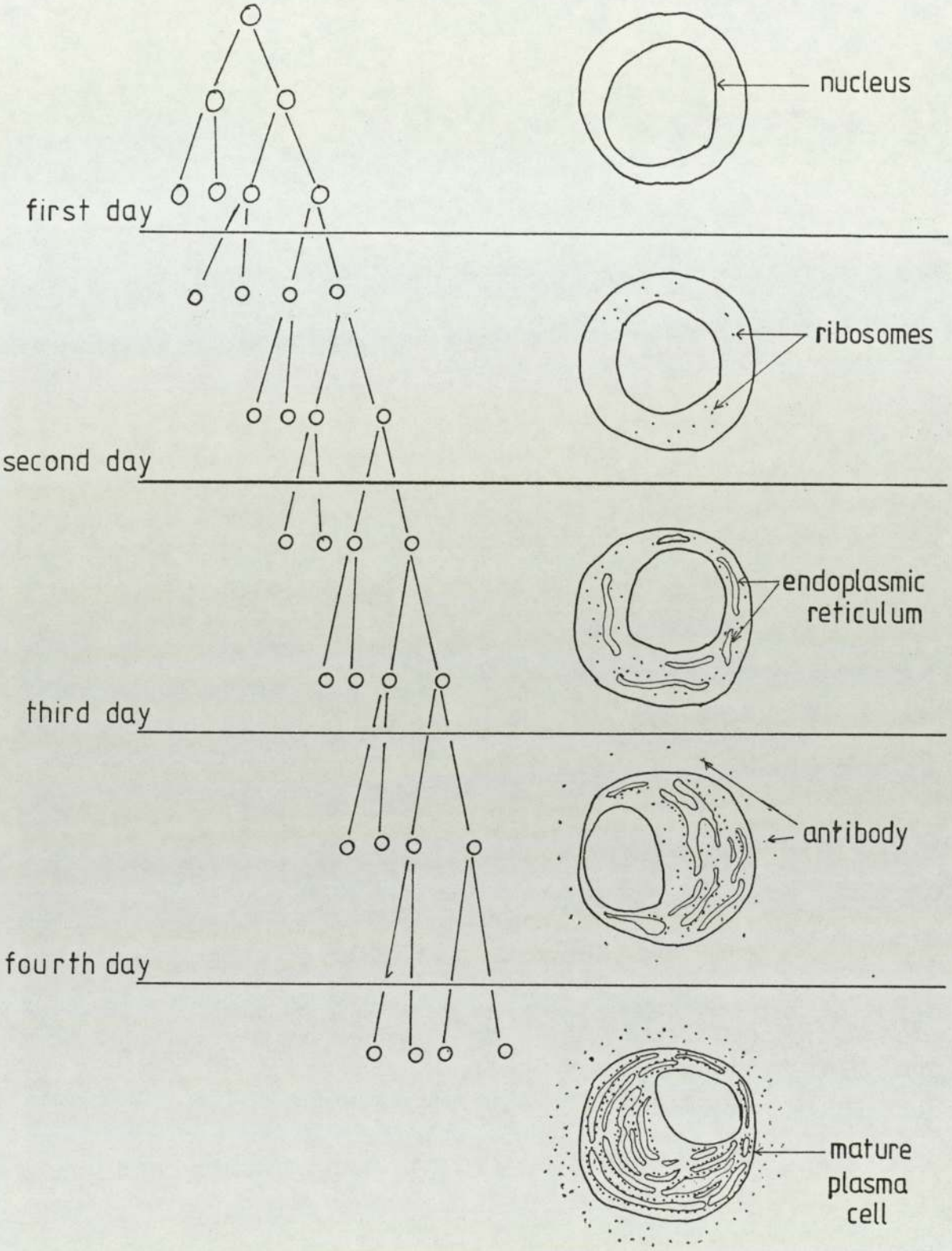


Figure 1.10 The clonal development of a selected B-cell as it differentiates into a plasma cell. Following antigenic stimulation, the plasmablast takes about 10 hours before it divides for the first time. (continued over).

Figure 1.10 (continued). The time between divisions gets progressively longer as the cells differentiate further but by the fifth day about 8-10 divisions will have occurred. This constitutes a 250-1000 fold amplification of the reactive members of the virgin B-cell population. An extensive endoplasmic reticulum and many ribosomes develop until by the fifth day antibody secretion reaches a maximum.

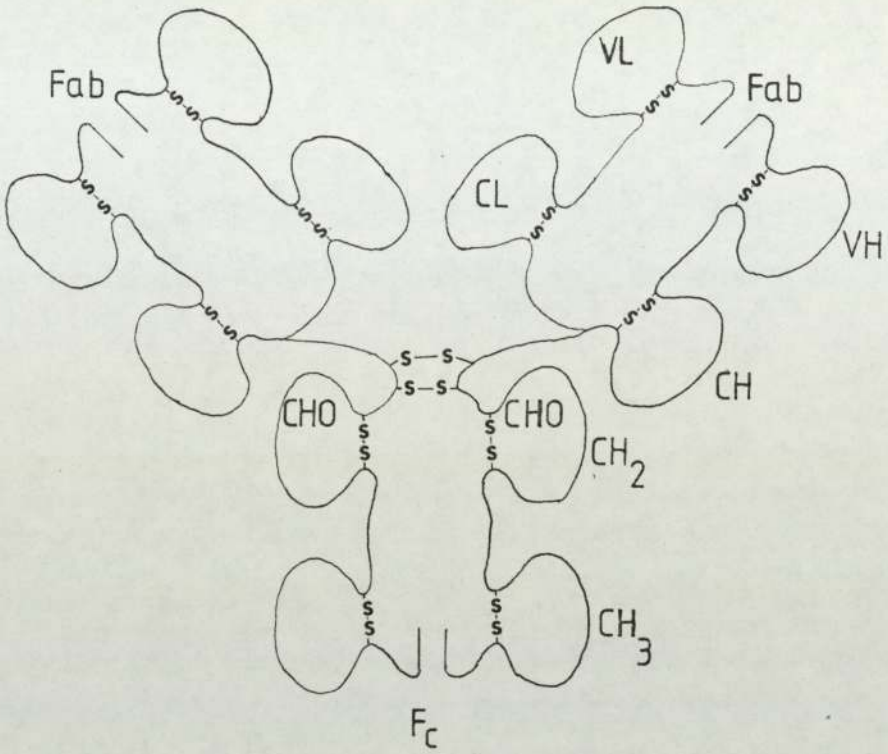


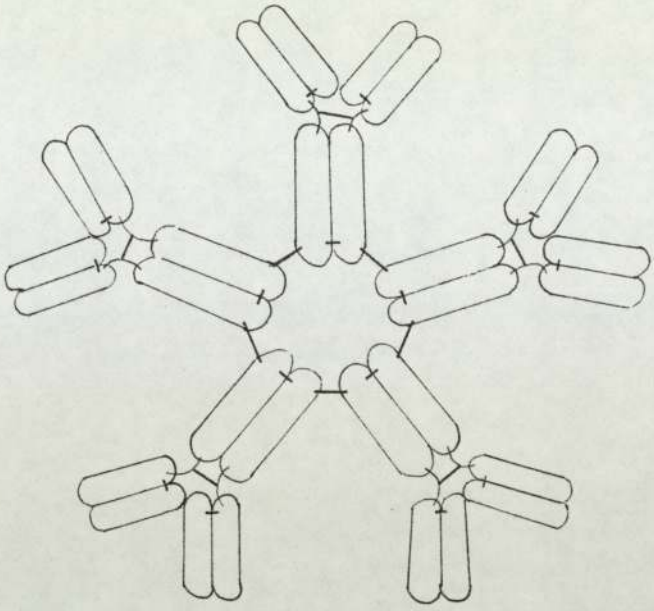
Figure 1.11 A representation of the IgG molecule. The molecule consists of 4 polypeptide chains two heavy (H) and two light (L) joined together by disulphide bonds (-S-S-). The constant regions of the heavy and light chains are designated CH and CL respectively and the numbers refer to the domain number. The antigen binding (Fab) and crystalizable (Fc) fragments are produced by papain cleavage. Epitope specificity is determined by the 3-dimentional structure of the antigen binding region of Fab. This structure depends on the primary sequence of the variable portions of the heavy (V_H) and light (V_L) chains. Receptors for complement are in the CH_2 region.

the tips of the 'Y' and it is by this part of the molecule that the antibody binds to the antigen. The Fc portion of the immunoglobulin molecule is thus presented away from the antigen surface. The epitope specificity of the antibody molecule is determined by the variable region of the Fab fragment. The structure of IgM is something like a pentameric form of IgG (fig.1.12). There are five 'Y' pieces joined together by a polypeptide chain; the J chain. The larger size of IgM means it sediments more quickly on density gradient centrifugation (19s compared with 7s for IgG) and it is thus sometimes referred to as "macroglobulin". The larger number of antigen binding sites on this immunoglobulin result in the molecule being more effective at cross linking between antigen particles than IgG. For this reason mixture of specific IgM with the relevant antigen results in rapid agglutination of the antigen.

In isolation, the only property of the immunoglobulin molecule is to show specific binding to a particular antigen which may agglutinate as a result of cross linking of binding sites. The exposed Fc portion of the molecule, however, confers biological significance upon the binding of antibody to antigen. B-cells, activated T-cells, macrophages, monocytes and polymorphs all possess membrane receptors for Fc and so can be recruited to sites where antigen is combined with antibody. Another very important function of the Fc portion of the immunoglobulin molecule is the binding and activation of complement.

The complement system consists of a series of eleven proteinaceous enzymes which can be destroyed by heating to 56°C for 30 minutes. The first unit C1 (which consists of several sub-units) must bind to two adjacent complement binding sites on bound immunoglobulin molecules if the reaction is to proceed. When bound thus, the reaction continues with the binding of further units of

IgM



IgG

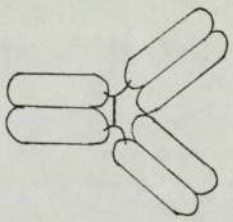


Figure 1.12 A comparison of IgM and IgG. The structure of IgM approximates to a pentameric form of IgG. The larger number of antigen binding sites per molecule make IgM more efficient at cross linking antigens and thus causing their agglutination. The presence on one molecule of adjacent complement-binding sites means that IgM is more efficient at complement fixation: it takes two IgG molecules close together to provide a suitable complement binding site. If the immunoglobulin is specific for heterologous red blood cells, these characteristics mean that IgM is a more powerful haemagglutinator and, in the presence of complement, a more powerful haemolysin than IgG. Diagram drawn to scale. (Adapted from Mezger,1970 and Green,1969).

complement from the surrounding fluid. While the C1 unit remains bound to the immunoglobulin the other units activated by C1 assemble on the surface of the antigen. If the antigen is a cell (e.g. an erythrocyte), the activated units insert themselves in the membrane to form a hollow 'doughnut' shape. The centre of this cluster is hydrophilic and allows the free passage of water and ions which flow into the cell until it bursts (Mayer,1973). Thus one bound C1 unit can result in the production of several cytolytic enzyme complexes which shower onto the surface of the antigen around the site of the bound immunoglobulin. The complement system is an example of a non-specific amplification mechanism.

Only the IgG and IgM classes of immunoglobulin can bind complement. Since IgM has five complement binding sites on each molecule, one IgM molecule is sufficient to provide the two adjacent sites necessary for activation of C1. In contrast IgG has only one binding site per molecule. Thus to activate C1, two IgG molecules must lie adjacently on the cell surface. Clearly IgM binds complement more efficiently than IgG. If the immunoglobulin is directed towards foreign erythrocytes, IgM would thus be a more powerful haemolysin than IgG.

In summary, lymphocytes are cells which are central to immunology. The two main sub-populations of lymphocytes T-cells and B-cells are further sub-divided into several functional groups and maturation stages. Lymphocytes are normally quiescent but when stimulated by antigen, and perhaps other (non-specific) agents, are capable of clonal proliferation to become effector cells. Products of both T-cells and macrophages can act as supplementary mitogenic signals in a co-operative interaction of T-cells, B-cells and macrophages and the immune response as a whole is under complex control. Finally of the immunoglobulin secreted from the mature

plasma cell, IgM is more efficient as an agglutinating antibody and in binding complement.

Some of the properties of lymphocytes, of immunoglobulin and of complement have been described above. Each has features which are designed to counteract the presence of antigen. Although emphasis has been placed on those features which allow amplification of the antigenic stimulus, any biological amplification system must clearly have a negative feedback system. The achievement of an optimum immune response requires the interaction of the components resulting in a finely controlled response which works both to amplify the initial antigenic stimulus and to suppress inordinate production of antibody. The true complexity of intercellular control of the immune response, which is discussed in the next chapter, has not yet been fully realized and remains an exiting and challenging area for research.

1.7 Cell co-operation and the Control of the Immune Response.

The tissues of the immune system are thus dispersed throughout the body connected by lymphatics and the blood circulation. The lymphocytes are the hub of the system and perform the role of both sensor and effector units. Unlike most physiological systems the cells of the immune system are not bound by connective tissue into organized structures. Their association with primary and secondary lymphoid tissues is loose and often transitory, and large numbers of cells are in circulation around the body. Yet the shifting units do not respond in isolation to the presence of an antigen but rather, produce a co-ordinated and controlled response involving co-operation between lymphoid cells which thus results in an effective immune response. The exquisite control of the immune system is without doubt one of its most intriguing properties. Direct neuronal control is largely useless to such a dispersed tissue. It is not surprising therefore to find that the cells of the immune system are subjected instead to all kinds of humoral control mechanisms, the complexity of which is only just being realized. One of the characteristic features of cell-mediated immune responses is their localized nature. Of course, this makes good physiological sense; there would be little point mounting a systemic reaction to an infected scratch on the left knee! If the co-operative responses are to occur only in the immediate vicinity of the antigen, it follows that the most effective humoral co-ordinating factors will also be localized. Thus much of the intricate cellular control of the immune response occurs within a microclimate around the antigen and the lymphocytes.

The clonal selection and proliferation of a sub-population bearing surface Ig with an epitope specificity for a determinant on

the antigen, must occur before appreciable specific antibody can be secreted. Antigens, such as LPS, are capable of stimulating B-lymphocytes to divide without the presence of any other stimulating factors. However, for the majority of antigens mere binding to the surface of the B-cell does not provide sufficient stimulus for cell division and the presence of T-cells is also required. Antigens requiring the presence of thymic lymphocytes are called thymic dependent (TD) antigens while LPS is an example of a thymic independent (TI) antigen. It is difficult to see quite what determines whether or not an antigen will be TI, but most are simple polymers such as polyamines or polysaccharides like LPS (Mitchel, 1974; Basten & Howard,1973). Also TI antigens often have a limited epitope heterogeneity and are capable of multipoint high avidity binding to Ig receptors on lymphocytes. (Wilson & Feldman,1972; Klaus,1975). The antibody response to LPS consists mostly of IgM of relatively homogeneous (low) affinity (Cosenza, Quintans & Lefkovits,1975; Baker, Prescott, Stashak & Amsbaugh,1971) which perhaps represents the baseline response which clones of B-cells can generate in the absence of co-operation though the baselines will vary between TI antigens.

The dependence of the responses to most antigens on the presence of a functional thymus has been demonstrated by experiments in which the immune response was first ablated by exposure to ionizing radiation or neonatal removal of the thymus and then reconstituted by transferring cells from syngeneic donors. It was found that following either neonatal thymectomy (NTx) or adult thymectomy (ATx) and irradiation, intact and functional B- and T-cells were required for the complete restoration of the primary immune response to SRBC (Claman, Chaperon & Triplett,1966; Miller & Mitchel,1968; Mitchell & Miller,1968). Although T-cells were essential for this result, they

were not the source of antibody (Nossal, Cunningham, Mitchell & Miller,1968). Thus T-cells perform a "helper" function, which has been shown to be mediated by a soluble dialysable factor (Feldman & Basten,1972; Feldman,1972).

In order that the T-cell can 'help' the B-cell response to an antigen and promote the B-cell proliferation, it must have some means of recognizing the antigen. The method of T-cell recognition has been a controversial subject for some time. It has been postulated that the receptor is of immunoglobulin type, a monomeric form of IgM which was designated IgT to show it was the T-cell immunoglobulin. Most groups have failed to demonstrate surface Ig on T-cells however, and only one group (Vitetta, Bianco, Nussenzweig & Uhr,1972) can consistently do so. An alternative postulation is that the T-cell antigen receptor is complexed from smaller entities. Thus Wigzell (1976) has suggested that the receptor consists of a complex of two heavy chains (coded for in the H-2 region) and two light chains of β_2 microglobulin. Antigenic specificity would be conferred onto this molecule by the binding of a small portion of the variable portion of immunoglobulin to the H2: β_2 microglobulin complex. At present it is not possible to reach a firm conclusion but there is good evidence that the antigen binding site on T-cells is either the same as, or shows a high degree of cross reactivity with the antigen receptor on the B-cell (Katz,1977). Whatever the nature of the T-cell antigen receptor, its antigen specificity allows particular T-cells to respond to an antigen in much the same way as B-cells and although T-cells never secrete antibody themselves they do have a profound influence on B-cell function.

Experiments with small molecular weight substances, called haptens, which by themselves will not initiate an immune response, have provided useful information about the recognition sites of

T- and B-cells. If a hapten e.g. trinitrophenol (TNP) is conjugated to a carrier protein such as ovalbumen (OA) or bovine serum albumen (BSA), the antibody formed is hapten-specific. In a mixed population of T- and B-cells the B-cell binds to the hapten portion of the hapten-carrier complex. The T-cell, meanwhile, binds to the carrier protein. A secondary (IgG) response to hapten-carrier can be produced after priming the animal with the same hapten-carrier complex. When T-cells from an animal sensitized to OA-TNP were mixed with B-cells from an animal sensitized to BSA-TNP, no secondary response occurred. However, the secondary response could be restored by including OA in the culture medium (Dutton, Falkoff, Hurst, et al., 1971). This shows that the co-operating T-cell is responding to different surface epitopes on the antigen, and that the soluble factor produced is not in any way specific for the hapten (fig.1.13).

A third cell, the macrophage, was introduced to the phenomena of cell co-operation when it was shown that the dialysable factor from activated T-cells was cytophilic for macrophages and that the putative IgT-macrophage complex could substitute for T-helper activity (Feldman & Basten,1972a). This observation could be of considerable significance to the in vivo immune response. Macrophages are known to be primarily responsible for the "trapping" of lymphocytes in spleen or lymph nodes (Frost & Lance,1974) so would be well placed for the presentation of antigens to B-cells while at the same time cytophilically binding the T-cell receptors and thereby providing the extra stimulus to B-cells necessary for activation (fig.1.14). There are few immune responses in vitro which are not affected by the removal of macrophages. Thus the primary humoral immune response to SRBC requires macrophages (Unanue,1972) but there is some disagreement over the rôle it has to play, if any, in the secondary response (Pierce,1969; Feldman & Palmer,1971).

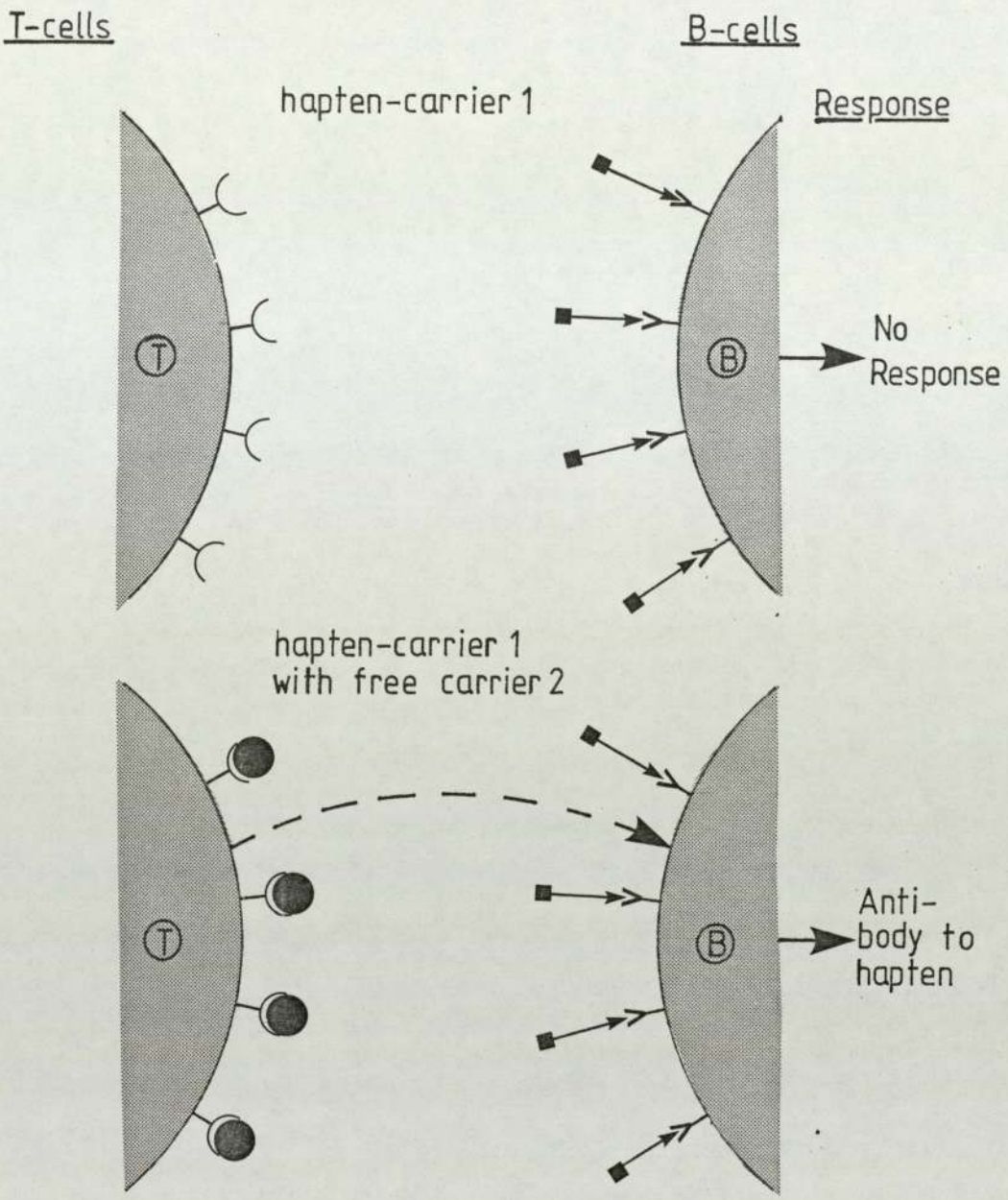


Figure 1.13 A possible mechanism for non-specific cell co-operation. Although both T- and B- cells have surface receptors, the cells 'recognize' different determinants on the same antigen. Using the hapten-carrier system, the T-cell is specific for carrier while the B-cell is specific for hapten. A secondary response to hapten will only occur if the carrier is the same as in the priming dose. Alternatively unconjugated priming carrier included in the medium will activate the carrier-primed T-cells which in turn non-specifically stimulate B-cells bound to antigen. The antibody produced in this situation is hapten specific.

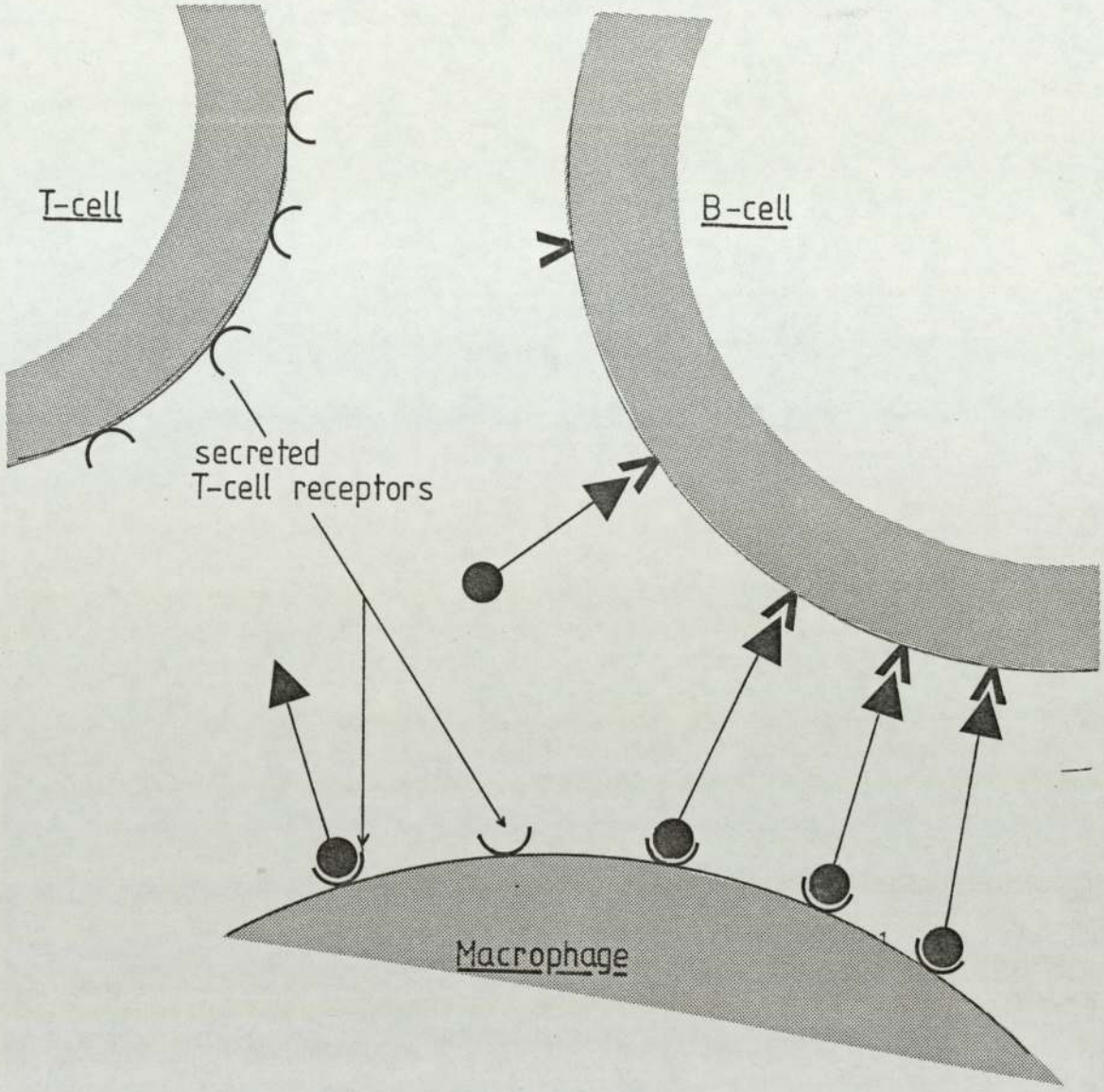


Figure 1.14 The possible mechanism of cell co-operation involving the presentation of antigen to B-cells via macrophage. Activated T-cells can release the surface receptors which are cytophilic for macrophages to which they bind. Macrophage: T-cell receptor complex can completely substitute for T-cells with respect to B-cell activation and antibody secretion.

The cell mediated immune (CMI) responses result from the effects of T-cells and phagocytes rather than B-cells. Here too co-operation takes place, usually in the form of T:T cell interaction. Certain parallels exist between the humoral (T:B cell co-operation) and the CMI (T:T cell) responses. Thus the T-effector cell (T_E) recognizes and binds to different surface epitopes on the antigen than does the T-helper cell (T_H) (Raff,1970). The epitope to which T_E responds is called the serologically defined epitope (SD) while the T_H cell responds to the lymphocyte defined epitope (LD) (Bach, Bach & Sonel,1976). Thus the T_E cell recognizes and binds to epitopes on the antigen which differ from those which are perceived and bound by the T_H cell. A typical assay of CMI response involves the measurement of radioisotope released from foreign 'target' lymphocytes as they are lysed by T-cell action. This phenomena is called cell-mediated lympholysis (CML) and it was found that both T_E and T_H cells were required to generate high numbers of CML effector cells (Asofsky, Cantor & Tigelaar,1971). On the whole the T_H cells belong to the Ly 1 and the Ly 2,3 sub-population (Cantor & Boyse,1975) but this is not, unfortunately, a fixed rule as the effector cell in the delayed hypersensitivity response is Ly 1 (Cantor & Boyse,1976). It now appears that the T_H-cell elaborates a soluble substance which can substitute for the presence of the helper cell (Plate,1976).

Once activated, the T_E cell undergoes the metabolic changes characteristic of a cell about to enter DNA synthesis. Prior to entering DNA synthesis however, the activated T-cell may secrete one or a number of soluble mediators of CMI which have been given the generic name of "lymphokines" (Dumonde, Wolstencroft, Panayi, et al.,1969). Since none of the lymphokines has been chemically identified, they can only be described by their biological activity.

It is known that they are produced from activated T-cells in the G_1 phase of the cell cycle. A large number of lymphokines have been described (Valdimarsson,1975; Morley, Wolstencroft & Dumonde,1978) and are probably of prime importance in the modulation of the immune response to antigen. Thus Macrophage Migration Inhibition factor (MIF), the best characterized lymphokine, probably serves to recruit macrophages to the site of infection. A number of mitogenic factors have also been reported. These include Antigen Independent Mitogenic factor (MF), Lymphocyte Transforming factor (LTF) and Antigen--dependent B-lymphocyte Mitogenic factor (LMF). Another lymphokine of interest is Osteoclast Activating factor (OAF) which induces local bone resorption (Raisz, Trummel, Mundy & Luben,1975) and may be responsible for the induction of a localized high Ca^{++} concentration, particularly next to the bone compartment.

The individual capabilities of the various sub-population of lymphocytes are thus optimized by co-operation with other lymphocytes. There is a clear division of labour in the responses of lymphocytes to each antigen into effector and accessory cells. The accessory cells can have a helper or suppressor rôle. Furthermore, in CMI responses factors secreted by activated T_E-cells serve to recruit more lymphocytes and macrophages into the microclimate thus created. The CMI response thus proceeds locally which is perhaps as well because extensive damage could be caused to the tissues of the body by some of the non-specific cytotoxic substances released.

Taking into consideration the number of amino acids involved in the antibody - combining site, a theoretical number of 10^{20}- 10^{30} different antibody specificities is possible. However, it is most unlikely that this number is produced and probably 10^6- 10^7 different specificities would be able to provide adequate recognition of nearly all antigens. There may well be a great deal of redundancy and

perhaps only a fraction of the total number of antibody specificities is probably ever used in the lifetime of an individual. One interesting model suggested by Jerne (1973) proposed that the immune system operates as a network of interacting units. This theory extended the idea of two or three sets of lymphocytes co-operating in a controlled immune response and allows a glimpse at the immune system as an integrated whole (fig.1.15).

The tenet of the theory is that immunoglobulin, whether surface--bound or free, is designed as a recognition molecule whose specificity is conferred by the primary sequence of the hypervariable regions of the H & L chains. However, these immunoglobulins can recognize epitopes not only on antigens but also epitopes on other immunoglobulin molecules. This will also apply to T-cell receptors even though these may not be entirely immunoglobulin in nature. The epitopes of the antibody molecule occur on both the constant and variable regions. Since the pattern of the variable region epitopes are determined by the variability of the polypeptide chain, and this in turn is related to the epitope universe, there are millions of different epitopes. The set of such epitopes on a given antibody molecule was named the idiotype of that molecule. If antibodies produced by animal A are injected into B, animal B will produce antibodies against the idiotypic epitopes (or idiotopes for short) of the injected antibody molecules. This occurs even if A and B are syngeneic. Within one animal, the idiotopes occurring on one antibody molecule are recognized by combining sites on a set of other antibody molecules. Similarly the idiotopes on the receptor molecules of one set of lymphocytes is recognized by the combining sites of the receptor molecules of another set of lymphocytes. Thus within one animal there may be a network of lymphocytes and antibody molecules that recognize other lymphocytes and antibody molecules, which in turn recognize still others.

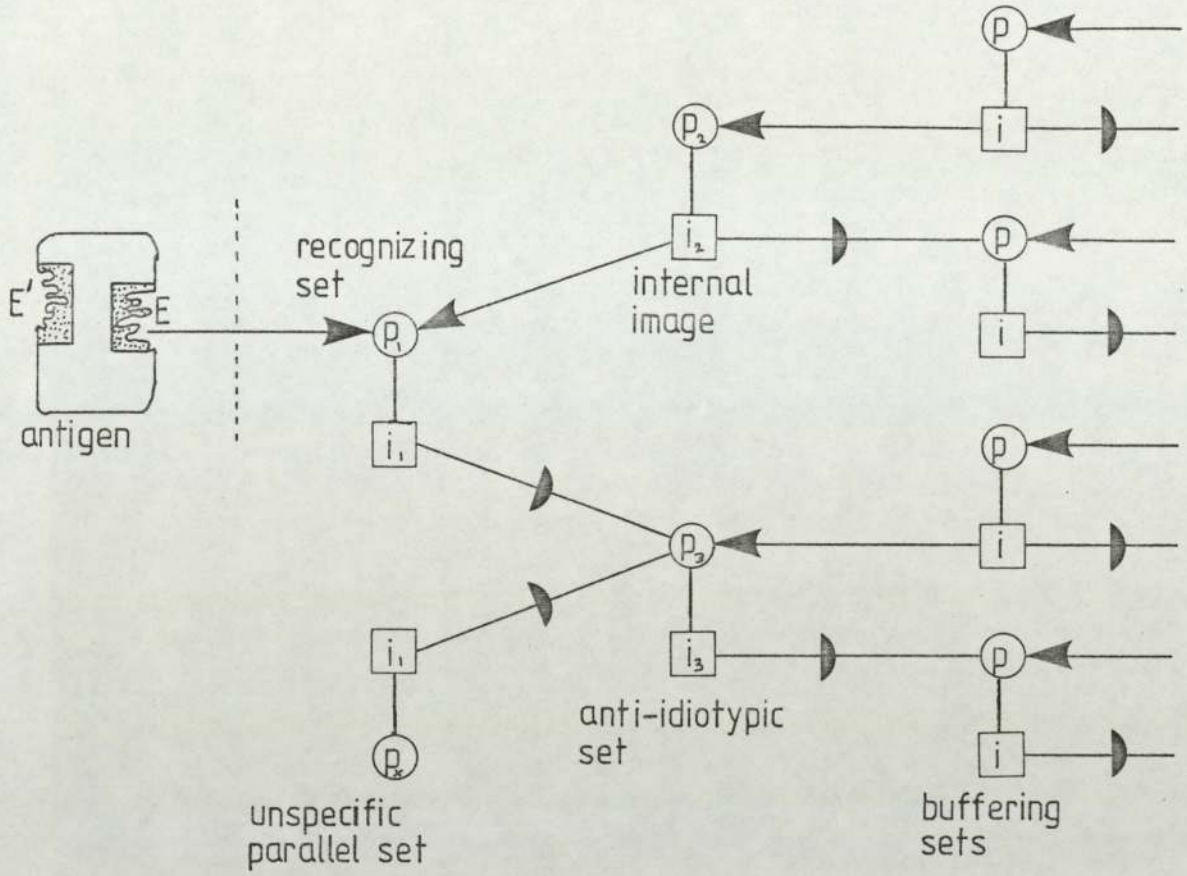


Figure 1.15 The integration of lymphocytes into a network. An epitope (E) on the antigen is recognized by a set (p_1) of combining sites on antibody molecules, both circulating antibody and cell--surface receptors. Cells with receptors of the recognizing set p_1 are potentially capable of responding to the antigenic stimulus (arrowhead) of epitope E, but there are constraints. The same molecules that carry combining sites p_1 carry a set of idiotopes (I_1). These are recognized within the system by a set of combining sites (p_3), called the anti-idiotypic set because they tend to suppress (bar) the cells of set I_1. (These idiotypes I_1 are also found on molecules with combining sites that do not belong to the recognizing set p_1, but rather are unspecific with regard to epitope E). (continued over).

Figure 1.15 (continued). On the other hand, the set p_1 also recognizes internal epitopes I_2, which therefore constitute an internal image of the foreign epitope E. In the steady state, molecules of the internal image set tend to stimulate cells of set p_1 and then to balance the suppressive tendency of the anti-idiotypic set. When the foreign antigen enters the system, its stimulatory effect on recognizing set p_1 allows cells of that set to escape from suppression. The resulting immune response to the antigen is modulated by the buffering effects of many more sets of combining sites and idiotopes (right) which have a controlling influence in the response.

The significance of this model is that it demonstrates that the properties of lymphocytes allow organization of the immune system into a balanced, self-regulatory whole. The normal equilibrium is upset by the presence of a foreign epitope. This provides additional stimulus to one population of lymphocytes, those with epitope-specific surface Ig or equivalent, which become activated and divide into a clone of effector cells. The balance between supression and stimulation from within the system is restored when the foreign epitope is removed.

Whether or not this network model is an accurate representation of the way in which the immune system functions remains to be clarified. However, there is supportive experimental evidence and it has two very attractive features. Firstly it explains a way in which the immune system can exhibit a burst of proliferative activity which culminates in the production of specific antibody and yet allows for the damping of the response (by anti-idiotypic suppressor sets) which must occur. Secondly, it illustrates how it is possible to construct, from existing knowledge of lymphocyte phsiology, an integrated system which exhibits many of the properties of the immune system.

Lymphocytes have been demonstrated to be very sensitive to a wide variety of humoral factors and not without good physiological reason for it is through humoral substances that most of the control of the immune system is exerted. It is now necessary to consider, in more detail the actual process of lymphocyte activation and associated biochemical events.

1.8 Lymphocyte activation: The rôle of Cyclic Nucleotides and their effect on the Immune Response.

Peripheral blood lymphocytes are normally in a quiescent state, in G_o, and have to be stimulated before they will divide. Such a population of cells have proved valuable for the study of the mechanism of cell activation. Under physiological conditions the stimulant would probably be an antigen. Within a population of lymphocytes from an unprimed animal very few would be activated by any single antigen. Only those which possess surface immunoglobulin receptor specific for an epitope on the antigen would respond. To obtain a large proportion of activated cells it has proved convenient, if less physiological, to use 'non-specific' lymphocyte mitogens.

Lymphocyte stimulation can be achieved by a variety of substances. They include plant extracts, the principle ones being an extract from kidney bean called phytohaemagglutinin (PHA) (Nowell,1960), concanavalin A (con A) from the Jack bean (Douglas, Kamin & Fudenberg,1969; Powell & Leon 1970), pokeweed mitogen (PWM) (Farnes, Barker, Brownhill & Fanger,1964; Borjeson, Reisfeld, Chessin, et al., 1966) and those derived from numerous other less well studied species (Douglas, et al.,1969; Barker & Farnes 1967). Other stimulants include bacterial lipopolysaccharide (LPS) (Andersson, Sjöberg & Moller 1972; Greaves & Janossy,1972) staphylococcal extracts, such as the exotoxin (Ling & Husband 1964), Hg^{2+} (Schoepf, Schultz & Gromm, 1967) Zn^{2+} (Ruhl, Kirchner & Bochert,1971), periodate (Novgrodsky & Katchalski,1971), antilymphocyte serum (ALS) (Grasbeck, Nordman & DeLa Chapelle,1963; Holt, Ling & Stanworth,1966), enzymes (Mazzei, Novi & Bazzi,1966), antigen-antibody complexes (Bloch-Shtacher, Hirshorn & Uhr,1968; Moller,1969) and lymphokines (Dumonde, Wolstencroft, Panayi et al.,1969). Of these PHA and con A have been most extensively studied.

If cultured without stimulant, lymphocytes remain quiescent but in the presence of PHA, large active cells appear with large nuclei and prominent nucleoli. On the third or fourth day of culture mitotic cells are to be seen. Small amounts of PHA are internalized during this process following capping and pinocytosis (Smith & Hollers,1970; Biberfield,1971; Taylor, Duffus, Raff & DePetris,1971). This is not thought to be relevant to the activation of the lymphocytes since PHA or PWM bound to sepharose beads (so that internalization is prevented) was still found to result in lymphocyte activation (Greaves & Bauminger,1972). A similar result was obtained with con A cross-linked to plastic petri dishes (Andersson, Edelman, Möller & Sjöberg,1972) or bound to acrylic polymer particles (Betel & Van den Berg,1972). The receptor sites for the PHA are thought to be glycoprotein rather than immunoglobulin.

Although the PHA is often described as a non-specific mitogen it, and the other mitogens mentioned above, do show a preference for stimulating particular sub-populations of lymphocytes even though equal amounts are bound by T- and B-cells. Thus while soluble PHA and con A are generally considered T-cell mitogens, (Stobo, Rosenthal & Paul,1972; Greaves, Bauminger & Janossy,1972) LPS predominantly affects mouse B-cells (Möller, Andersson, Pöhlit & Sjöberg,1973). Interestingly, B-cells are stimulated by PHA or con A if these are bound to insoluble materials (Andersson et al.,1972; Greaves & Bauminger 1972). Thus B-cells must have the appropriate receptors and this phenomenon might be related to macrophage presentation of antigen to the B-cell in a suitably immunogenic form.

Within minutes of addition of mitogen an increase in membrane fluidity occurs (Ferber, Reilly, de Pasquale & Resch 1974; Barnett, Scott, Furcht & Kersey,1974) and there is activation of membrane bound enzymes such as ATPases (Quastel & Kaplan,1971; Averdunk &

Hoppe-Seylers,1972) or lipid metabolising enzymes (Resch, Ferber, Odenthal & Fischer,1971; Ferber & Resch,1973; Ferber, Resch, Wallach & Imm,1972; Ferber, et al.,1974; Fischer & Mueller,1971). Phospholipid turnover also increases (Fisher & Mueller,1971) and there is a quantitative change in pinocytosis (Biberfeld,1971). There is an increase in normally weak adhesion of lymphocytes to glass culture vessels and to each other (Killander & Rigler,1965). The net surface charge on the lymphocyte has also been observed to change (Vassar & Culling,1964) though this does not occur if the non--agglutinating form of PHA is used (Caspary & Knowles,1970). There is also a change in the number of hormonal binding sites (Krug, Krug & Cuatrecasas,1972). In common with other activated cells, lymphocytes take up a variety of small molecules when stimulated. Following stimulation with PHA lymphocytes show an increased uptake of nucleosides and sugars (Peters & Hausen,1971a; b), the non-utilized amino-acid α-aminobutyric acid (Mendlesohn, Skinner & Kornfeld, 1971), phosphate (Cross & Ord,1971) and choline (Peters & Hausen, 1971) and there is also extensive disturbance of the ionic distribution. Thus following PHA stimulation there is an influx of K^+ (Quastel & Kaplan, 1971) Na^+ (Dent,1971) and Ca^{++} (Allwood, Asherson, Davey & Goodford,1971). Studies of the changes induced by PHA on the general metabolism indicate how far reaching are the effects of PHA stimulation since virtually every pathway investigated has shown alterations following stimulation. Thus elevation of RNA synthesis (Kay,1966), protein synthesis (Fillingame & Morris,1973), polyamine (Kay & Cooke,1971; Kay & Lindsay,1973) and carbohydrate metabolism (Peters & Hausen,1971a; Roos & Loos,1970) have all been observed. The rate at which glucose is taken up by the cell is an interesting anticipatory response since for a while it is accumulated prior to an increased rate of glycolysis (Peters & Hausen,1971a).

Not surprisingly, a large number of alterations in enzyme activities have been reported (Ling & Kay,1975) which presumably are not unassociated with the gross metabolic changes described above.

Some of these changes are undoubtedly not essential for blastogenesis to occur, but are 'associated' events. The presence of the PHA is required for a period of time for the induction of blastogenesis to occur but there is a point when the cell becomes committed to DNA synthesis and removal of the stimulant after this time does not prevent the cell proceeding to mitosis (Kay,1969; Younkin,1972). It follows that during that phase, the initial stimulus (PHA binding to surface receptor) becomes transduced to a cellular stimulus which is independent of the external stimulant. Since the cyclic nucleotides have been shown to have a dramatic effect on the proliferation of other cell types (section 1.4), attempts have been made to link the early events observed following PHA stimulation with cyclic nucleotide metabolism (and to try to measure any gross changes in cyclic nucleotides or their relevant cyclases or phosphodiesterases). Much of this work was summarized in section 1.4 and it remains to reiterate some salient points and present a working hypothesis of the possible mechanism of lymphocyte activation.

The importance of ionic flux which follows membranal stimulation has been amply demonstrated. Thus removal of calcium from the medium during the first 12 hours after PHA prevents DNA synthesis (Whitney & Sutherland,1973). Also, the calcium ionophore A23187 which allows calcium to flow into the cell (down its concentration gradient) mimics the effects of PHA stimulation and results in the initiation of DNA synthesis (Luckason, White & Kersey,1974). Similarly the influx of potassium can be prevented by treating with the glycoside ouabain which is a potent inhibitor of Na/K ATPase (Post, Merris,

Kinsolving & Allbright,1960; Dunham & Glyn,1961). If human peripheral lymphocytes are activated with PHA they will incorporate uridine into RNA and thymidine into the DNA which is synthesized. The inclusion of ouabain almost completely inhibits this incorporation (Quastel & Kaplan,1968; Kasakura,1969) thus indicating a possible rôle for K^+ in the induction of blastogenesis.

At present the influx of calcium ions is the most likely candidate for the most important single event (other than stimulant binding to receptor) in the triggering process. Calcium is known to stimulate lymphocyte guanylate cyclase (Katagari, Terao & Osawa, 1976). Thus an influx of calcium leading to guanylate cyclase activation is consistent with the observed rise in cGMP occurring after stimulation of peripheral blood lymphocytes with con A (Coffey et al.,1977). Furthermore cGMP is known to stimulate a number of events associated with mitogenesis.

It must be remembered that any hypothesis is at this stage partly hypothetical. The search for a single "second messenger" may to some extent be misguided. Once the stimulator (e.g.PHA) has bound to its membrane receptor the number of effects begin to increase as a sequence of biological amplification steps occurs. The only single event might be PHA and receptor binding ! Thereafter the initial stimulus manifests itself in a variety of ionic fluxes and membrane associated effects all of which may activate different pathways within the cell. Thus the elevation of cGMP may be one of a number of divergent events, none of which alone, result in cell activation but rather act in concert to this end.

A considerable amount of work has been done on the possible rôle of cyclic nucleotides on the immune response. The effect of these substances on lymphocyte transformation may or may not have relevance to the immune response which involves many steps in addition to the

early proliferative phase. The results obtained are far from unequivocal but vary depending upon the test system studied, the dose and type of antigen and the dose of nucleotide used.

An assay for cell-mediated immunity is the cytotoxic assay where a target cell, perhaps a tumour cell line, is lysed by the action of activated lymphocytes. Death of the target cells is often measured by the release of a radioactive label (Cr^{51}) previously incorporated into the cell cytoplasm. Using this assay it was observed that agents which stimulated the elevation of endogenous cAMP (e.g. prostaglandin, cholera toxin or theophylline), resulted in reduced lymphocyte cytotoxic action. In contrast, cholinergic agents, known to stimulate the accumulation of cGMP in other *in vitro* assays, caused an enhancement of cytotoxic action towards the target cells (Strom *et al.*,1972). Cyclic AMP is known to inhibit the secretion in a number of tissues such as lung (Kalinger, Orange & Austen,1972), in leukocytes (Lichtenstein & Margolis,1968) and from platelets (Jost & Rickenberg,1971). It seems likely, therefore, that in the cytotoxicity assay it is a secretory process which is inhibited by cAMP. T-cells can be said to have three functions; a helper function in antibody production; the production of soluble mediators of CMI; cell mediated cytotoxicity. However, not all these functions are equally affected by cAMP. Thus while abrogation of the lytic effect is achieved by treatment of lymphocytes with cholera toxin, which stimulates adenylate cyclase thus elevating endogenous cAMP, the production of lymphokines such as MIF and LT is unaffected (Heney *et al.*,1974). This can be interpreted to support the notion that the capacities for cytotoxicity and lymphokine secretion lie in different sub-populations of T-cells. Alternatively the cytotoxic and lymphokine production may be in the same cell but be totally unrelated biochemically and lymphokine production is cAMP-independent.

Following immunization with SRBC a very rapid rise in cAMP concentration occurs in the spleen (Plescia et al.,1975; Yamamoto & Webb,1974). The peak value occurs only 2 minutes after challenge and reaches values 2-4 fold higher that basal. The effect is transitory for levels drop below normal 20-60 minutes after challenge and remain thus for a protracted period. The concentration of cGMP in the spleen also changes but in a different pattern. Thus 5-10 minutes after challenge cGMP levels rise gradually and remain higher than background for up to seven days (Yamamoto & Webb,1975). The immunological significance of these changes in not clear. It has been suggested that for B-cells antigen-receptor interaction activates adenylate cyclase and the ensuing rise in cAMP leads to paralysis of the lymphocyte. However, in the presence of T-cells a stimulus is provided (the 'second stimulus') which activates guanylate cyclase (Watson,1975). Thus in a model somewhat akin to the Goldberg model for fibroblasts (section 1.3) the ratio of cAMP: cGMP becomes regarded as the important regulatory factor, rather than absolute amounts. Antibody forming cells (AFC) precursors stimulated by antigen in the absence of T-cells become paralyzed because of the rise in cAMP. In the presence of helper T-cells however, the additional stimulus lowers the cAMP: cGMP ratio and allows lymphocyte induction to proceed.

Thus the activation of lymphocytes by mitogen or antigen is heralded by a number of observable phenomena; changes in membrane permeability, ionic distribution and enzyme activity. The precise rôle played by cyclic nucleotides is debatable but it is quite clear that these substances are able to modify the reaction of lymphocytes to activating stimuli. Lymphocytes are known to have a number of surface hormone receptors (Melmon, Weinstein, Shearer, et al.,1974). considering the known influence which hormones have on cellular

cyclic nucleotide concentration it is not surprising that hormones influence the expression of immunity and that these effects are almost certainly mediated by altered cyclic nucleotide metabolism. The next secion will consider the effect of hormones on the immune response.

1.9 Hormones and the Immune Response.

It has been amply demonstrated that cyclic nucleotides and their dibutyryl derivatives have profound effects on cell metabolism and proliferation (section 1.3). Though they may not have the universal second messenger rôle which was originally ascribed to them, levels can often be induced to alter following stimulation of the cell. Furthermore induction of elevated endogenous nucleotide levels (e.g. with theophylline or cholera toxin) results in altered cell metabolism. The action of some hormones is known to be mediated by the cyclic nucleotides so the finding that lymphocytes possess surface receptors for a number of hormones (Melmon, et al.,1974) suggested the possibility that hormones may modulate lymphocyte metabolism. This was confirmed by experiment both in vivo and in vitro. Particular attention has been paid to the 'tissue hormones' and prostaglandins. There is a close correlation between the ability of these hormones to inhibit the immune response and to elevate cAMP levels. One problem in studying these hormones however, is that it is difficult to assess precisely what are the tissue concentrations of the active substance. It is thus not possible to determine what actually constitutes a physiological dose and thus the physiological significance of the results. However, it has been shown that histamine, β-adrenergic amines and prostaglandins (E_1 and E_2) all lead to an inhibition of the production of antibody cells (Melmon, Bourne, Weinstein, Shearer, Kram & Bauminger,1974). In addition histamine, catecholamines and prostaglandins have been shown to inhibit the IgE-mediated release of histamine and other mediators of inflammation (Ishizaka, Ishizaka, Orange & Austen,1971; Assem & Schild,1969) and specific cytolytic activity of mouse splenic lymphocytes

(Lichtenstein, Henney, Bourne & Greenough,1973; Henney, Bourne & Lichtenstein,1972). All three hormones act via cAMP (stimulating its production) which seems to be acting in leukocytes and mast cells as an inhibitory second messenger (Melmon, et al.,1974a).

Prostaglandins (Pgs) do not accumulate intracellularly but are synthesized and released in response to varied and often unknown stimuli. Many cells can synthesize and secrete Pgs including several components of the immune system. Thus PgE is released by mitogen and antigen stimulated leukocytes in culture (Ferraris & DeRubertis, 1974), and stimulation of human eosinophilic leukocytes results in synthesis and release of PgE_1 and E_2 from these cells (Hubscher, 1975). During phagocytosis, rabbit polymorphonuclear leukocytes (PMN's) release PgE_1 which is chemotactic to other PMN's (McCall & Youlten,1974; Higgs, McCall & Youlten,1975). Also macrophages (Morley,1974; Gordon, Bray & Morley,1976) as well as non-specifically activated peritoneal exudate cells (Bray, Gordon & Morley,1974) have been proven to be sources of Pgs. Furthermore leukocytes almost certainly contain receptors for Pgs and are thus likely physiological targets for Pgs (Lichtenstein,1973).

A number of immunological effects of Pgs have been observed both in vivo and in vitro. Exogenous Pgs (particularly of the E series) have been demonstrated to increase or decrease intracellular cAMP in a number of tissues (Butcher & Baird,1968; Smith, Steiner, Newberry & Parker,1971; Smith, Steiner & Parker,1971; Bourne, Lichtenstein, Melmon, et al.,1974; Whitfield, et al.,1973). PgF_2 has been shown to increase cGMP levels in rat uteri (Kuehl, Cirillo, Ham & Humes,1973). However the mediation of the effects of Pgs on lymphocytes by the cyclic nucleotides remains to be resolved.

The humoral antibody response is decreased on treatment with PgE_1 (Zurier & Quagliata,1971) and both PgE_1 and E_2 cause

accumulation of cAMP and inhibit the activity of plaque forming cells (PFC) in three different strains of mice (Melmon, Bourne, Weinstein, et al.,1974a). The PHA-induced transformation of murine lymphocytes, a T-cell response is inhibited more than 50% by PgE_2 while it has no effect on blastogenesis stimulated by the specific B-cell mitogen derived from pokeweed (Stockman & Mumford,1974). Exogenously administered Pgs have been reported to result in enhanced graft-versus-host reaction and in prolonged skin allograft survival (Loose & DiLuzio,1973; Anderson, Newton & Jaffe,1975; Quagliata, Lawrence & Phillips-Quagliata,1973) and Pgs of the E series inhibit the in vitro cytotoxic reaction of primed cells to allogeneic thymus cells (Strom, Deisseroth, Morganroth, et al.,1974). PgE is capable of suppressing the secretion of inflammatory mediators by mast cells in anaphylactic reactions and inhibits lymphocyte participation in delayed hypersensitivity reactions (Bourne, et al.,1974). PgE_1 also reduces the production of macrophage migration inhibition factor (MIF) a lymphokine released from stimulated T-cells, thereby affecting macrophage activity (Bray, et al.,1974; Gordon, et al.,1976).

It has been suggested that the pathological state e.g. in rheumatoid arthritis may he represented by defective lymphocyte reactivity towards E-type prostaglandin. The absence of an effective negative feedback to the lymphocyte would lead to continued production of both lymphokines and prostaglandins, both of which have potentially pathogenic effects (Morley, 1974).

As described previously (section 1.6) the thymus plays a critical rôle in the development of the immune system. The development of pre-T-cells into mature T-lymphocytes may well be influenced by local humoral factors produced and active within the thymus and whose target cell is the immature T-cell. Such a hormone might be called a differentiating hormone. A variety of evidence

has been produced which supports the notion that the thymus can also act in a truly endocrine fashion. The endocrine system tends to function as a web; each member affecting and being affected by other members of the system. This is probably because of the presence of numerous feedback loops which result in a reverberating stimulus through several parts of the endocrine system.

Neonatal removal of the thymus (NTx) affects the functioning of the hypophysis and in particular the degranulation of the STH producing cells (Pierpaoli & Sorkin,1968; Bianchi, Pierpaoli & Sorkin,1971). The results suggest the possibility of a reciprocal relationship between thymus and hypophysis. In neonatal females, thymectomy has been shown to sometimes result in absence of ovarian follicles and corpora lutea and the interstitial cells become hyperplastic (Nishizuka & Sakakura,1969; 1971a;1971b). The operation also delayed puberty and slowed maturation of ovaries and uterus (Basedovsky & Sorkin,1974). The nude mouse, which is genetically athymic, has reduced plasma T_4 concentrations and shows marked hyperplasia of the zona reticularis of the adrenal (Pierpaoli & Sorkin,1972a; 1972b). It must be remembered however that these last effects may not be due solely to the athymic condition but may be related to other defects of this genetic mutant. However the effect of thymectomy on other endocrine tissues infers that there could be a physiologically important substance secreted from the thymus.

The immunological effects of thymic deprivation are now well documented. Thymectomy can be performed in the adult when it is usual to include sub-lethal irradiation to kill peripheral lymphocytes and followed by repopulation of the animal with bone marrow. If performed in the neonate, no supplementary irradiation is required. Either of these treatments results in a reduction of

peripheral and thoracic duct lymphocytes. Specific 'T-dependent' areas of the spleen and lymph node become hypoplastic and there is a general impairment of immune responsiveness. Thus skin graft rejection and delayed hypersensitivity are retarded, wasting disease may set in and antibody responses are diminished apparently because of loss of T_H cells since the numbers of antibody producing cells remain the same (Miller & Osoba,1967).

The *in vitro* behaviour of lymphocytes derived from NTX animals generally confirm the *in vivo* findings. They show reduced mixed lymphocyte reaction (Takiguchi, Adler & Smith,1971) reduced PHA responsiveness (Meuwissan, vanAlten & Good,1969) and reduced graft versus host response (Miller & Osoba,1967).

The results obtained following adult thymectomy (ATx) without any irradiation are less clear-cut. The impairment of allograft rejection did not always occur (Miller,1965) though after a variable, extended time period post operatively, conventional antigenic challenges also elicited reduced responses (Metcalf,1960; Taylor, 1965; Miller, deBurgh & Grant,1965),

The major support for the notion that the thymus may perform an endocrine rôle in the immune system came from studies of either cell--free extracts or from thymus grafts in cell-impermeable diffusion chambers. Thus the defects which occur following NTx could be eliminated by implanting living thymus tissue in a cell-impermeable chamber. The decline in peripheral blood leukocytes was halted and the humoral axis of the immune response, measured by levels of anti-heterologous protein antibody, was restored. The helper T-cell (T_H) function in the response to sheep erythrocytes (SRBC), assessed by levels of haemolysin, haemagglutinin and numbers of plaque forming cells, was also restored. Similarly defects of the cell-mediated immune response (CMIR) found in NTx animals i.e. responses to skin

allografts, xenogeneic tumour grafts, delayed hypersensitivity and graft versus host, are all prevented by such implants (Trainin & Small,1973; Trainin,1974).

Although some doubt has been cast on whether the chambers used were truly impermeable to cells the finding that extracts from thymus mimic the results is further strong supportive evidence that the thymus produces humoral factors which are of physiological significance. Thus, cell-free extracts of thymus increase the number and mitotic activity of splenic lymphoid follicles, prevents wasting and increases lymph node weight and ^{3}HTdR incorporation. The rejection of 1st or 2nd set allografts or allogeneic tumours is enhanced by repeated injection of crude thymic extract into NTx mice. The GvHR of mice is repaired and there is a slight increase in the haemolysin production by SRBC-injected NTx mice which has been attributed to replacement of T_H function (Trainin & Small,1973; Trainin,1974; Luckey,1973; Stutman & Good; Goldstein & White,1973).

The thymus may well produce a group of hormones. Several active factors have been isolated by different methods and may represent either different forms of one hormone or a group of related hormones. All are polypeptides but the molecular weight varies between 1000-12,000D. Thymic Humoral Factor (THF) (Kook, Yakir & Trainin, 1975) has a molecular weight of 6000D when crude and, if purified, this drops to 3,220D. THF may represent an active sub-unit of Thymosin which is the extract produced by Goldstein (Goldstein, Thurman, Cohen & Hooper,1975) having a molecular weight of 12,200D. Other factors isolated are Thymic Factor (Bach, Dardenne, Pleau & Bach,1975) which has a M.W. of 1,000D, and Thymopoietin (Goldstein, 1974) with a M.W. of 7,000D. Although these substances have different molecular weights and are isolated by different procedures

they form a group of substances with many physiological actions in common.

The target cell for these substances has not been identified with certainty. THF may be a lymphopoietic hormone controlling differentiation of lymphoid cell line by inducing stem cells to divide and possibly also reduce the rate of division of more mature T-cells concomitantly with the aquisition of immunocompetant properties. THF stimulates adenylate cyclase in splenic lymphocytes resulting in accumulation of endogenous cAMP and these effects are independent of protein synthesis (Kook & Trainin,1974; 1975). If splenic lymphocytes from A strain mice are cultured with lymphocytes from A x B strain mice (F_1) a one way mixed lymphocyte reaction occurs. The A strain lymphocytes are activated by the AB strain cells while the latter are unable to reciprocate. If the A strain lymphocytes are obtained from NTx animals no response will occur. However, substances which elevate cellular cAMP such as theophylline, DBcAMP or PGE_2 confer reactivity to the spleen cells from NTx animals. The effect of these compounds mimics THF which causes a rise in cAMP which is an obligatory event in the process of induction of immune competence. Thus the ability of spleen cells to respond to allogeneic stimulation was abolished when the increase of cAMP was prevented, while the immune competence of spleen cells from intact mice was not impaired under this condition (Sutherland & Robinson,1968).

The incubation of thymocytes or spleen cells from NTx animals with THF was also accompanied by a reduction in DNA synthesis and cell division which is consistent with the other studies of the effects of cAMP on thymocytes and lymphocytes (section 1.3). These results suggest that by preventing cell proliferation THF, via elevated cAMP, stimulates protein synthesis and induces the cells to

acquire immunocompetence allowing the otherwise immature spleen cells from neonatally thymectomized mice to respond to the *in vitro* GVH assay.

In contrast to polypeptide hormones which have receptors on the surface of the cell, the receptors for steroid hormones are usually cytoplasmic. Although the steroids are not classically connected with the cyclic nucleotides they have marked effects on the immune system.

The adrenal corticosteroids are generally lympholytic, particularly in high doses. These effects were most striking in new-born mice where a single injection (0.25mg) of cortisol acetate administered neonatally produced runts with atrophic thymi and spleens about one fifth normal weight (Schessinger & Mart,1964). The cells are destroyed at metaphase so that it is the rapidly dividing and/or immature lymphocytes which are affected (Dougherty, Berlinger, Schneebeli & Berlinger,1953, Weissman,1973). Lymphocytes were observed to shed 'blebs' in cytoplasm shortly following corticosteroid treatment (Dougherty, *et al.*,1953) which eventually caused cytolysis *in vivo* and *in vitro* (Trowell,1958; Cohen & Claman, 1971). These effects have dire immunological consequences. Thus the rejection of allografts between outbred guinea pigs (Sparrow, 1953) or rabbits (Billingham, Krohn & Medawar,1951) is prolonged by injections of cortisol acetate and the ability of regional lymph nodes to respond to such a challenge is also abrogated (Scothorne, 1956). The physiological significance of these experiments must be questioned because of the high doses used and because it has been shown that at lower doses corticosteroids can be stimulatory (Ambrose,1976). Also in pharmacological concentrations glucocorticoids blocked macrophage migration inhibition activity by directly antagonizing the inhibitory effects of MIF on the

macrophage (Balow & Rosenthal,1973).

Like the corticosteroids the sex steroids are immunosuppressive. Removal of the gonads, like removal of the adrenals, results in marked hypertrophy of the lymphoid organs (Trowell,1958). Various authors have reported reduced immune responses in females compared to males. Progesterone and methoxy progesterone acetate suppressed the antibody formation in response to BSA and synthetic progestogens of the 19-nor group prolonged allograft survival (Hulka, Mohr & Liberman,1965). Repeated administration of oestrone to guinea-pigs during the delayed hypersensitivity reaction to tuberculin or thyroglobulin was found to reduce induration while both antibody titres and the erythematous reaction remained unaffected (Kappas, Jones & Roitt,1963). Similarly the administration of testosterone to rats reduced the incidence and severity of adjuvant-induced arthritis and the development of autoallergic thyroiditis (Kappas, et al.,1963).

A convincing link between the immune response and somatotrophic hormone (STH or growth hormone) has also been demonstrated. Thus in Snell-Bragg mice, a strain with hereditary recessive pituitary dwarfism, the thymus is severely atrophic and shows a marked reduction of mitotic activity. The spleen is similarly affected with striking hypotrophy of the lymphoid tissue surrounding the germinal centres. Variable effects were observed in the (sternal) bone marrow from moderate to severe depletion of cells and reduction of both granulopoietic and erythropoietic activity. The Snell-Bragg mice also showed a much reduced immune response to SRBC, producing only about one fifth the normal numbers of plaque forming cells (Baroni,1967). These conditions can be ameliorated by replacement therapy with STH (Pierpaoli & Sorkin,1971; Pierpaoli, Baroni, Fabris & Sorkin,1969). The deficient syndrome can be created in normal

mice by hypophysectomy (Pandian & Talwar,1971) or by the injection of anti-STH serum (Pierpaoli & Sorkin,1968).

Other hormones are also known to affect lymphoid tissues. Thus thyroxine is known to influence lymphocyte development (Fabris, et al.,1971; Gyllenstein,1962) while insulin stimulates thymocyte proliferation in vitro (Pandian & Talwar,1971) and pancreatectomy leads to a reduction of thymic weight (Pierpauli, Fabris & Sorkin, 1970).

The apparent importance of the glucocorticoids and the sex steroids in control of the immune response should be treated with caution. Much of the work done was with pharmacological doses of hormone so that any effect obtained might be not relevant to a normal immune response. The ablation of the appropriate glands may provide a clearer picture of the physiological rôle played by them, even if it does not necessarily indicate a direct action of a particular hormone. Of the few experiments performed on the effect of adrenalectomy alone, this operation failed to alter the survival time of allografts in mice (Medawar & Sparrow,1956; Graff, Lappe & Snell,1969) or xenografts in rats (Bilder,1976). Similarly, neither thyroidectomy nor hypophysectomy altered the initial rejection time of rat xenografts.

There are thus a number of immunological phenomena which are affected by hormones acting either alone or in conjunction with other hormones. If physiological significance is to be ascribed to a particular response then careful consideration must be given to the dose of hormone used. Often experimental conditions adopted include pharmacological doses of the hormone (e.g. the in vivo corticosteroid experiments) so that the results obtained have little or no relationship to 'normal' responses. This need not, however, undermine the value of the result providing that no attempt is made to equate pharmacology with physiology.

As the preceding literature has shown, lymphocytes are extremely sensitive to the humoral and ionic environment and hormones, ions and the cyclic nucleotides are inexorably woven into the control of both their division and differentiation. The humoral consequences of parathyroidectomy have been discussed elsewhere (section 1.4). Briefly, the removal of the parathyroids causes an immediate and lasting drop in plasma total and ionised calcium which has wide ranging consequences reflecting the importance of the calcium ion *in vivo*. The maintainance of the calcium ion concentration appears to be necessary for the integrity of the thymus since this organ exhibits steroid-independent involution following parathyroidectomy (Perris, *et al.*,1970). This effect is reversible by reinstating normocalcaemia by treatment with either calcium chloride injections, parathyroid hormone (Perris, *et al.*,1970; Perris & Whitfield,1971) or feeding on high calcium/low phosphate diet (Robinson & MacIntyre, 1967). The parathyroidectomized (and therefore hypocalcaemic) animals also shows increased susceptibility to wasting following irradiation, and hypoplasia of lymphoid tissues (Rixon & Whitfield, 1972). The observation that in normal mice injections of calcium chloride or calcitonin affected the immune response to sheep erythrocytes (Braun, Ishizuka & Seeman,1970) indicated that the symptoms of parathyroidectomy may have immunological significance.

The purpose of this study was to discover the possible relationships between the parathyroid glands and the immune response.

Section 2.

Methods & Results.

2.1 Characteristics of the Immune Response in Rats.

That the higher vertebrates require a constant internal environment in order to survive has become axiomatic. Any change within that environment is restricted by a variety of homeostatic devices. Examples of the control of plasma constituents such as a glucose or calcium following a change in their normal concentrations spring readily to mind. Perhaps less obvious are the changes designed to maintain cellular homeostasis; the loss of blood cells or lymphocytes via haemorrhage or chronic thoracic duct drainage and the loss of liver cells via partial hepatectomy elicits the proliferation of progenitor cells to re-establish the cellular status quo. The"effector substance" which bridges the gap between stimulus and response is frequently humoral and may involve the increase in the concentration of a stimulator (hormone) or a decrease in the concentration of an inhibitor (chalone). On occasion however, cellular proliferation may be in excess of the requirements for the maintainance of cell numbers in the interest of overall bodily integrity. This is particularly evident following antigenic infringement when both virgin and memory T- and B-cells respond to antigen by clonal proliferation and differentiation into a discrete population of effector cells which eliminate the foreign element. Although antigen alone can trigger lymphocyte proliferation *in vitro* it does not rule out the possibility of some additional humoral mitogenic influence, in addition to that provided by the antigen, amplifying lymphocyte numbers *in vivo*.

Many physiological conditions characterized by an elevated mitotic activity are also associated with hypercalcaemia which parallels and is essential for the proliferative increase (section 1.4) Briefly, these include the elevated proliferative activity of the

bone marrow following haemorrhage, during pregnancy and following cobaltous chloride injection. Heightened mitotic activity in the young rat and the variations which occur during oestrus cycle and in a circadian fashion are likewise paralleled by plasma calcium changes. Since simple injections of calcium chloride can alone cause elevated bone marrow and thymus mitotic activity, the hypercalcaemia which accompanies elevated proliferation assumes potential physiological significance.

The first series of experiments were designed to discover if the proliferative events of the immune response were also associated with an hypercalcaemic phase *in vivo*. Rats were chosen as the experimental animal primarily because the calcium homeostatic system can readily be manipulated (see section 2.2), something which has proved more intractable in the mouse.

Male rats of the Wistar strain were used in preference to females which are known to exhibit variation in plasma calcium during the oestrus cycle (Smith & Perris,1976). Water and a standard laboratory diet (Spillers 41B) were supplied *ad libitum*. The temperature was a constant 18-20°C and lighting was normal daylength supplemented with incandescent light until 17.00 hours in mid-winter. All animals weighed between 225-250g thus avoiding the juvenile elevation of plasma calcium and its associated heightened mitotic activity (Perris, Whitfield & Tölg,1968).

To measure the proliferative phase of the immune response antigens were administered intraperitoneally (i.p.) in 0.9% saline between 9.00 and 10.00 hours. The day of injection was designated day 0. Samples were collected on subsequent days also between 9.00 and 10.00 hours which thus avoided errors due to circadian variation in plasma calcium concentration and proliferative activity (Hunt & Perris,1974). Blood samples were obtained under ether

anaesthesia and the animals were allowed to expire under ether before removal of any other tissues.

The first antigen chosen was sheep red blood cells (SRBC) which elicits a co-operative immune response during which both B- and T-cells respond (Miller & Mitchell,1968; Katz & Benacerraf, 1972). The primary response to SRBC in mice (and presumably rats) also requires the presence of macrophages (Unanue,1972). This requirement for macrophages probably also exists during the secondary response to SRBC (Feldman & Palmer,1971) so that the sheep erythrocyte represents an antigen likely to stimulate a general immune response.

Sheep red blood cells (SRBC) (Flow Laboratories) were stored in Alsever's solution (a buffered glucose-citrate solution) at $4^{o}C$ until required and were used within three weeks of storage under these conditions. Immediately before injection the cells were washed three times in isotonic saline by employing on each occasion centrifugation at 800 x g and gentle aspiration of the pellet in fresh saline. The final pellet was resuspended in nine times its own volume of saline which thus resulted in the formation of a 10% suspension of SRBC. The antigen was injected i.p. at a dose of 1ml. per 200g rat which was equivalent to $4x10^{9}$ cells/ml. Control rats received either saline alone or an equal number of isologous (rat) erythrocytes similarly prepared and administered.

At any one time within a population of dividing cells, there is a certain proportion in the mitotic (M) phase. Such cells are histologically recognizable because of the condensation of the nuclear material and its organization into prophase, metaphase, anaphase or telophase configurations. The number of cells in mitosis expressed as a percentage of the total population of nucleated cells is called the Mitotic Index (MI). Since cells only

spend a relatively short time in M, and not all the cells of a tissue may be actively cycling, only a small percentage of cells will be found in M at any time. The MI is thus only suitable for tissues with a high mitotic activity and large numbers of actively cycling cells. The thymus and bone marrow fall into this category.

Since the MI only measures the percentage of cells in M at the time of fixation it is possible for several artifacts to occur. The extrusion or destruction of large numbers of non-proliferating cells from the population would lead to an increase in the MI which would not reflect elevated proliferation. However, it has been shown that an increase in bone marrow MI following $CaCl_2$ injections was not associated with any reduction in the numbers of non-dividing cells (Perris & Whitfield,1971). Alternatively a prolongation of M without a proportional increase in the total cell cycle time would lead to an increased MI. This would be caused by the increase in time which a cell would take to pass through M. Thus at the time of fixation proportionally more cells would be found in M. Although this remains a theoretical possibility, most cell cycle studies reveal that such prolongation of the M phase occurs only very rarely (Mitchison,1971). Nevertheless MI data revealing apparent increased mitotic activity should preferably be confirmed by alternative techniques.

On the other hand, a shortening of the overall cell cycle time without a decrease in M would also lead to an increased proportion of cycling cells in M at a given time i.e. an increased MI. This would represent a genuine increase in the rate of production of new cells. Alternatively recruitment of cells from G_o population would increase the numbers of actively cycling cells from which it follows that the proportion of cells entering M would increase correspondingly. The recruitment of cells from a G_o, or restricted

G_1 compartment is the most likely explanation for the increased proliferative activity of lymphocytes observed in this study since these cells are known to be activated from such a condition upon antigenic contact (Ling & Kay,1975).

Given the above assumptions, use of the MI provides a quick means of assessing any increase in proliferation relative to control animals. It has the added advantage that there is no intervention by the experimentor prior to the termination of the experiment. Artifacts are thus minimal and cells found in M would certainly have complete dividing had the experiment not been terminated. Thus use of stathmokinetic agents such as colchicine was neither practical nor desirable for the experiments performed. Colchicine has pronounced hypocalcaemic effects in vivo (Heath, Palmer & Aurbach, 1972) and was thus totally unacceptable for this series of experiments which demanded careful plasma calcium titrations. Furthermore, termination of experiments at 9.00 hours prohibited the 6 hour accumulation of metaphases required to obtain a satisfactory result with this agent.

Methods in which either tritiated or iodonated nucleotide precursors were used proved insufficiently sensitive (see appendix 1). Autoradiographic methods were used with success in both bone marrow and in spleen, which has a much lower proliferation rate. The preparation required is, however, much more tedious than that for MI so that for bone marrow the latter was used in preference though autoradiography was used for confirmatory purposes.

To assess the MI of the bone marrow the left femur was removed, cleared of adherent muscle, washed and the epiphyses removed with clean bone cutters. With care the bone marrow plug could be completely removed intact by placing a pasteur pipette filled with 1ml. of saline in the lower cut end and squeezing the bulb gently.

Once the main plug was removed the hollow shaft was flushed through with clean saline. The plug was gently aspirated with a wide bore pasteur pipette in a total of 2ml of saline until a uniform, unicellular suspension was obtained. Frothing was avoided as this could have damaged cells due to shearing forces created at the surface of bubbles. Two drops of the cell suspension were mixed with one drop of calf serum (Wellcome) on a clean slide, spread and air dried in a current of warm air. When dry, the slides were transferred to 10% neutral formol saline solution for fixation for at least 30 minutes. The slides were rinsed briefly in water prior to staining in Delafields haematoxylin (Whitfield, Brohée & Youdale, 1964) dehydrating and mounting in Depex. When hardened sufficiently, the slides were observed under oil immersion at a magnification of 1250x and the cells in mitosis scored as a precentage of the population of nucleated cells. In practice it is very difficult to recognize early prophase from interphase so that for the purpose of this study M is defined as late prophase to telophase. A total of at least 2000 cells were counted by two separate observers from coded slides and any counts which did not closely agree were reassessed.

Using the above procedures, the MI of the bone marrow was assessed following challenge with 10% SRBC in saline. The results show (fig.2.1.) that the normal MI for adult male rat bone marrow is just under 1%. Following SRBC the mitotic index of the bone marrow increased from this basal level to 1.3% by the second day ($p<0.01$) and 1.4% on the third day, ($p<0.001$). The elevated mitotic activity fell rapidly back to levels not significantly different from basal on subsequent days. At its peak the mitotic index is an average of 46% greater than the control value.

Although the bone marrow performs an erythropoietic rôle in the adult rat, it also has an important function as a primary lymphoid

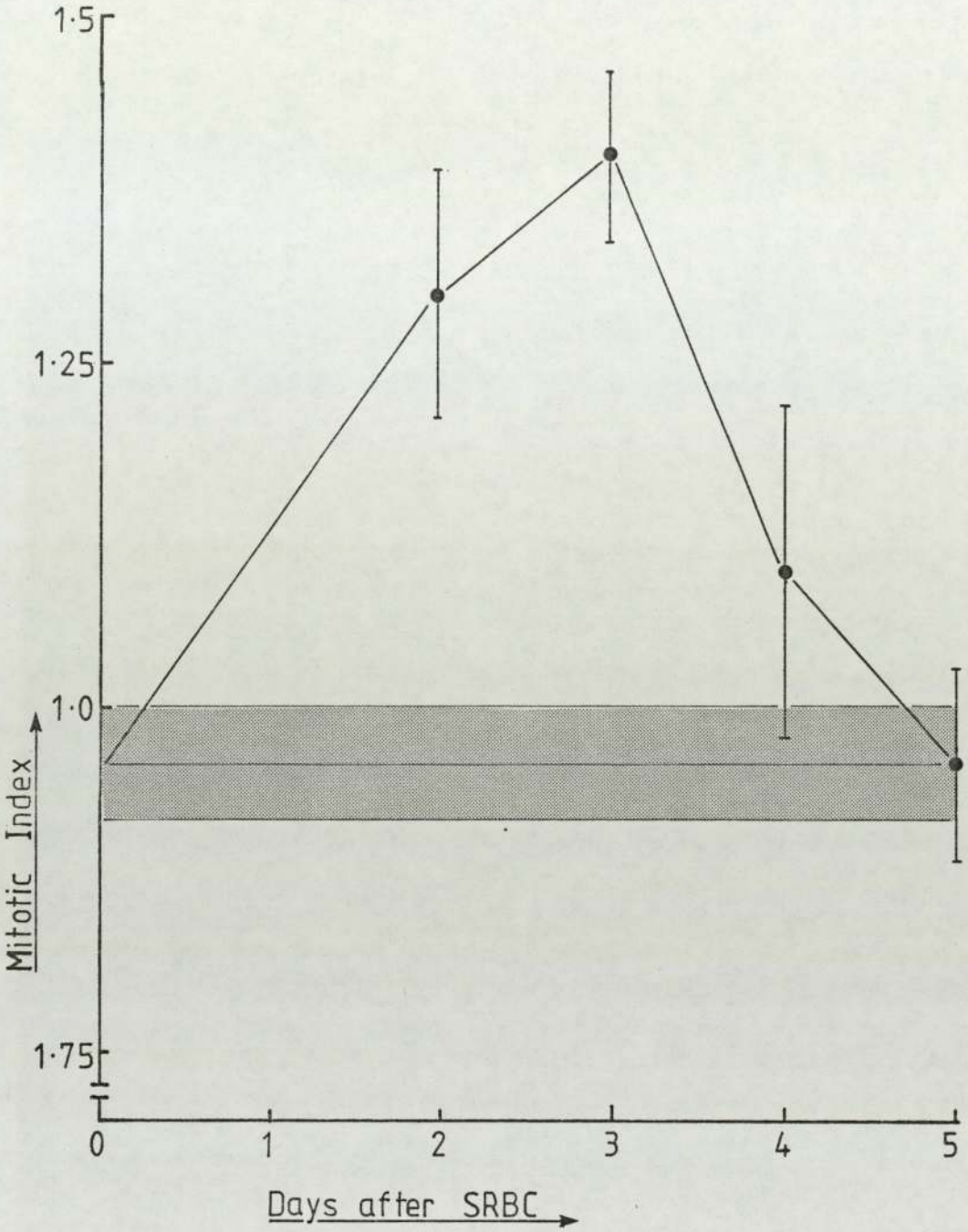


Figure 2.1. The effect of antigen on the Mitotic Index (MI) of the bone marrow. Rats were immunized i.p. on day 0 with 1ml per 200g rat of 10% sheep erythrocytes (SRBC) in saline and the MI assessed from bone marrow smears prepared on subsequent days. The proliferative activity rose steadily in the first few days rising to a peak on day 3 and thereafter declining rapidly to normal levels. The elevation over control values is significant on days 2 ($p<0.01$) and 3 ($p<0.001$) and at its peak the MI is 46% higher than in unchallenged animals. Values are means $\pm$ 1 S.E.M. of between 5 and 22 measurements.

organ. However, primary lymphoid tissue is not generally considered to be antigen sensitive since the precursor cells will not yet possess the relevant surface receptors. The proliferative response observed in rat bone marrow is thus open to at least two main interpretations. Firstly the rat bone marrow may have a considerable number of immunocompetent mature lymphocytes and thus perform the function of both primary and secondary lymphoid tissue. Alternatively the response of the bone marrow cells might be not driven directly by the antigen but rather by some other factor produced during the immune response.

At the time of sacrifice of these animals, a blood sample was taken by cardiac puncture for calcium analysis using a syringe with a 21 gauge needle. This was inserted through the diaphragm between the cleft of the xiphisternum and left rib-cage and pushed gently forward into the heart. Since no heparin was included in the syringe (as this could dilute the plasma) it was necessary to work quickly. A slight negative pressure applied to the syringe allowed blood to begin to flow when the point entered the ventricle. The syringe was allowed to fill with the minimum of suction thus reducing the possibility of haemolysis as the blood passes through the needle. Although 5ml can be readily removed by this method usually 2-3ml was all that was required and by collecting smaller quantities there was less risk of either clotting or trying to withdraw the blood too fast and causing haemolysis. After removal of the needle from the syringe the blood was transferred to a heparin coated sample tube (Teklab). Thus heparinised blood samples were obtained without resort to heparinised saline. The blood was mixed in the tube by gentle inversion and the sample centrifuged at 1000 x g to obtain the plasma which was then used for calcium analysis. Handled thus, there was no detectable haemolysis in the plasma. Although a small degree

of haemolysis did not interfere with the subsequent calcium titration, large amounts caused a marked depression of the end point, presumably due to quenching of the green fluorescent light by the red haemolytic products. Plasma samples were titrated for calcium within a few hours of collection, during which time they were stored at 4°C. Storage for longer periods at -20°C was not possible as this was found to result in the formation of precipitates and altered the calcium values from those obtained with fresh samples.

The apparatus used for the titration was a Corning Calcium Analyser (model 240) in conjunction with an SMI micro pipette capable of delivering 50µl or 100µl samples. The Calcium analyser is an automatic electronic burette which titrates total calcium against the calcium chelator ethylene glycol bis-tetracetic acid (EGTA). The end point is provided by an indicator, calcein, which fluoresces in u/v light in the presence of calcium. Thus the end--point was marked by a disappearance of fluorescence and was measured photoelectrically. Calcium concentration in mg per 100ml. of plasma (mg%) was displayed digitally to two decimal places. The type of pipette used (SMI micropettor) had a re-usable glass tip and teflon plunger which protruded beyond the tip when the sample was expelled. Thus there was no sample left adhering in the tip after expulsion and the very small amount of carry-over was eliminated completely by washing the tip twice in double distilled water between samples and drying with a tissue. The choice of this type of pipette prevented the errors known to occur with air/liquid interface pipettes when pipetting serum (Rideout,1975). Using this technique the values obtained for each sample were typically within 0.05mg% i.e. about $\pm$ 0.25% variation.

The effect on plasma total calcium of antigenic challenge on day 0 with SRBC is shown in figure 2.2. The mean basal calcium

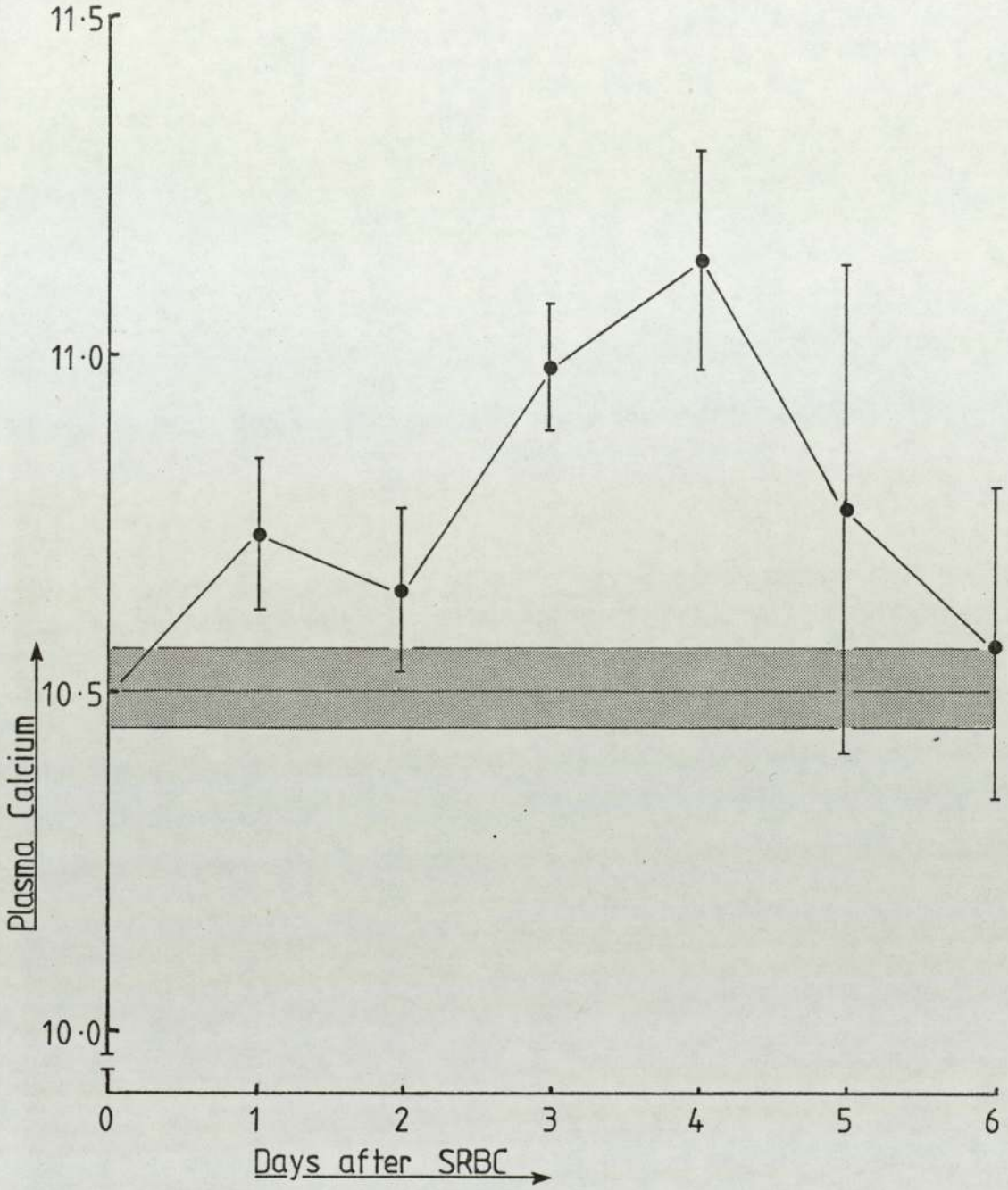


Figure 2.2. The effect of antigen on the plasma calcium concentration of normal rats. Sheep erythrocytes were administered i.p. in saline at a dose of 1ml. per 200g rat on day 0 and plasma calcium was measured from samples obtained by cardiac puncture. Plasma calcium concentration was significantly elevated on days 3 and 4 ($p<0.001$) following challenge. The rate of decline back to normal levels seemed to vary between different rats so that the variation in calcium concentration on day 5 is quite large. The points represent means and the vertical bars 1 S.E.M. of between 5 and 18 animals and the horizontal line shaded area is the mean $\pm$ 1 S.E.M. of 47 control animals.

concentration was found to be 10.5mg% ± 0.06 (S.E.M.) Following antigenic challenge the plasma calcium was found to rise to a peak value of 11.00 ± 0.09 mg% on the third day and to thereafter decline to normal levels on subsequent days. The hypercalcaemia persisted on the fourth day after challenge at about the same value (11.04 ± 0.16 mg%) but thereafter declined rapidly to normal levels on subsequent days. Although the rise to hypercalcaemia occurred fairly synchronously between rats, the decline was less so. This resulted in a greater variation of plasma calcium on the days after the peak of hypercalcaemia (day 3). The development of an hypercalcaemia thus parallels the bone marrow proliferation measured by mitotic index.

Since a complete response to SRBC, like other antigens, involves the co-operation of B-cells T-cells and macrophages (Katz & Benacerraf,1972; Unanue,1972) it is impossible to say which of these cell types might be responsible for development of the hypercalcaemia which is provoked. It was possible, however, to choose antigens which elicit greater or lesser T-cell co-operation (section 1.7).

Like SRBC, bovine serum albumen (BSA), elicits a co-operative response which results in the production of specific anti-BSA antibody. However, it is a soluble protein antigen unlike SRBC which is particulate and likely to be phagocytosed by peritoneal magrophages. BSA was administered at the dose of 5mg/Kg i.p. in saline. The proliferative response in the bone marrow followed a similar pattern to that which followed challenge with SRBC. Thus maximal proliferative activity was seen on day 3 (fig.2.3) and a significant hypercalcaemia had developed parallel to the elevated proliferation (fig.2.4).

An antigen derived from molluscs, called keyhole limpet haemocyanin (KLH), elicits a response which is heavily T-cell

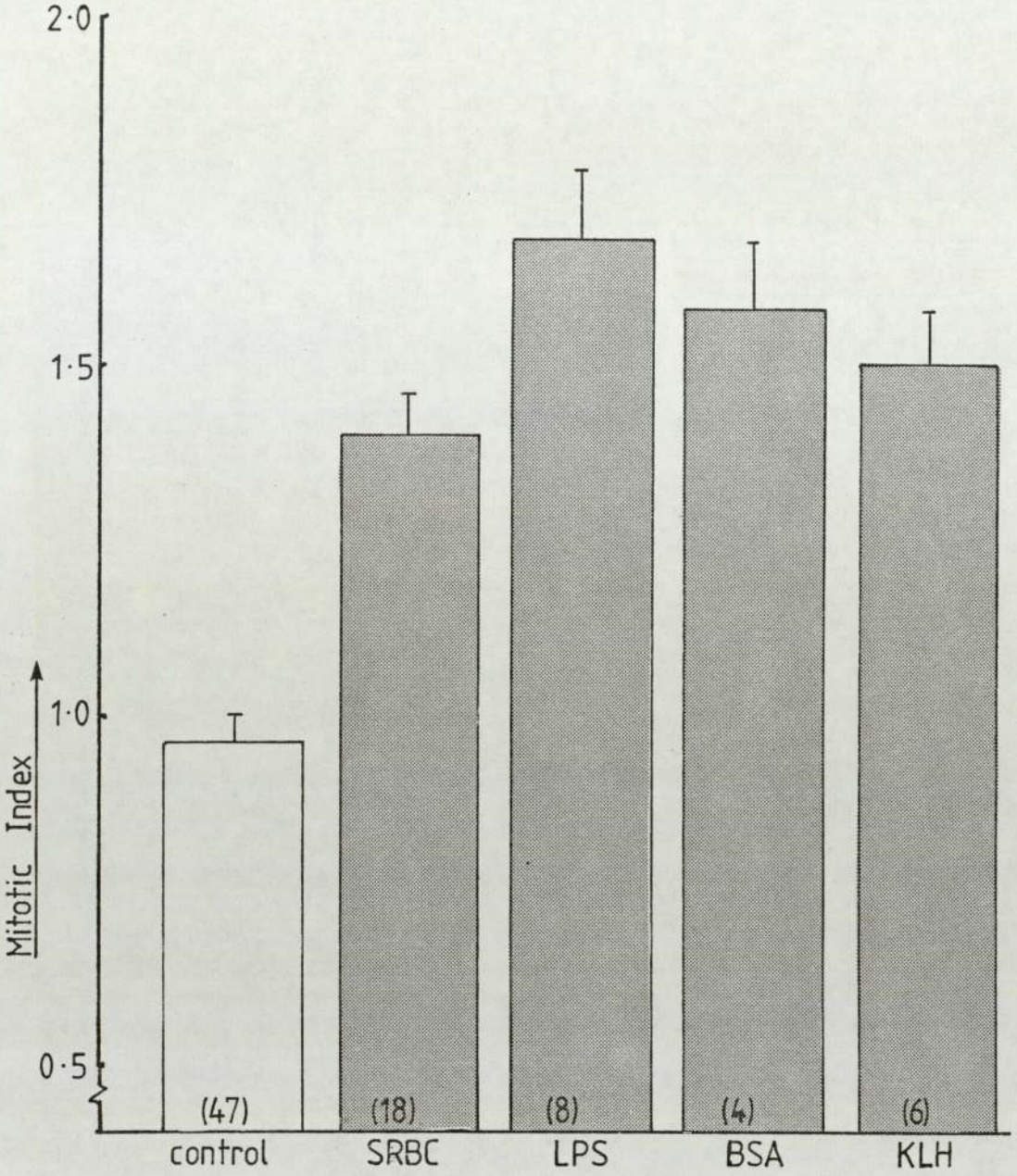


Figure 2.3. The elevation in bone marrow mitotic index following the injection of antigens. The maximum responses are shown and occurred on day 3 following sheep erythrocytes (SRBC), bovine serum albumen (BSA) and keyhole limpet haemocyanin (KLH) and on day 1 following bacterial lipopolysaccharide (LPS). Values are means ± 1 S.E.M. of between 4 and 47 animals as indicated in parentheses.

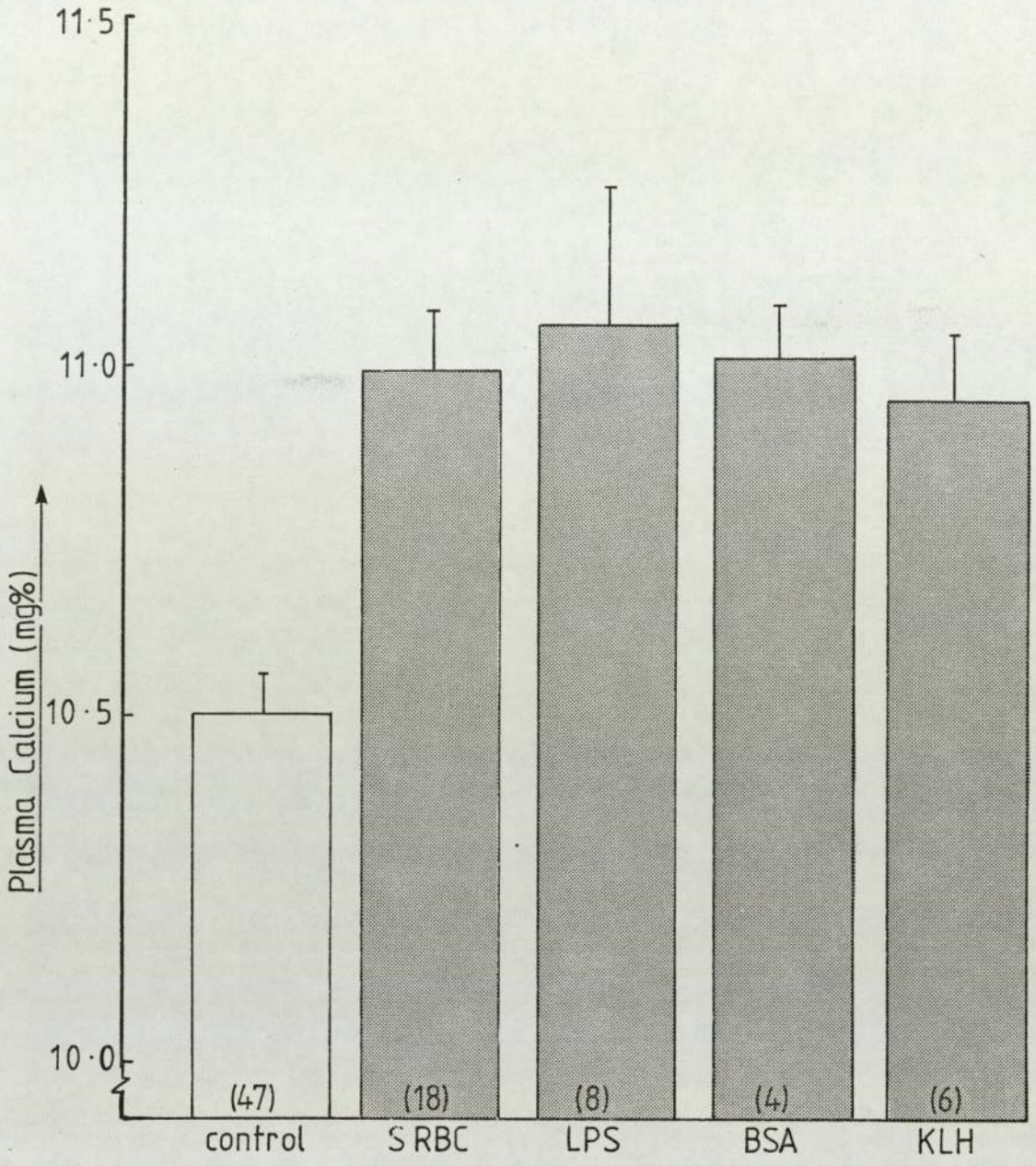


Figure 2.4. The plasma calcium concentration at the time of peak bone marrow proliferation following antigenic challenge with either sheep erythrocytes (SRBC), bacterial lipopolysaccharide (LPS) bovine serum albumen (BSA) or keyhole limpet haemocyanin (KLH). A significant hypercalcaemia developed in each case ($p<0.001$). Values are means $\pm$ 1 S.E.M. of between 4 and 47 animals as indicated in parentheses.

dependent. KLH, administered i.p. in saline at a dose of 1mg/Kg, resulted in the familiar peak of proliferation on day 3 (fig.2.3) associated with an hypercalcaemia on the same day (fig.2.4).

The rejection of non-isogeneic skin grafts is another immune response in which there is a large T-cell involvement. Thus presentation of a foreign skin graft results in activation of T-cells of various subsets notably T_C (cytotoxic) and T_H (helper) though there is undoubtedly B-cell activation also since antibodies to the foreign tissue are detectable following grafting. Furthermore there is considerable macrophage infiltration of the graft bed where they phagocytose target cells which have been coated with antibody from plasma cells (opsonised). Graft rejection thus involves the co-operation (see section 1.7) of nearly all cellular axes of the immune response. The ability to reject a skin graft donated by a different species (a xenograft) is thus a general test of cell mediated immunity (CMI) (see section 1.6).

To observe the response of rats to this type of antigenic challenge skin from mouse tail was grafted on to rat flank. The method used for this xenograft (Herbert,1978) was to aseptically remove the mouse tail skin which was then cut longitudinally and laid epidermis down in a dish containing sterile isotonic saline. The graft was prepared for transplantation by cutting circles of tail skin with a sterile cork borer. The graft site was prepared by shaving the flank of a rat under ether anaesthesia and cleansing the skin with alcohol. A circle of tissue was removed from behind the scapula over the ribs by lifting the epidermis with a pair of sterile forceps and cutting out the skin with a pair of sterile curved scissors held horizontally with the tips pointing upwards. The circular graft site thus produced, consisted of dermal tissue and was slightly larger than the donor graft. The graft was placed

on the recipient graft bed and covered first with sterile lint, and then vaseline soaked gauze finally the thorax of the rat was wrapped in a wet plaster bandage. When set, the plaster cast tended to restrict breathing but the animals could accomodate quite readily by switching to abdominal breathing. Grafts from rat tail to rat flank (isografts) were employed as controls.

Following antigenic challenge provided by the xenograft, once again an increase in both the mitotic activity of the bone marrow and the concentration of calcium in the plasma was noted (fig.2.5).

Bacterial lipopolysaccharide (LPS) from E. coli is a well known polyclonal B-cell activator in mice (Andersson, Sjöberg & Moller, 1972; Greaves & Janossy,1972). It exhibits both antigenic and mitogenic properties. Thus high affinity antibody is readily produced against it but some clones are activated non-specifically. These actions were reflected in the speed with which it stimulated proliferation in the bone marrow (fig.2.6). Thus, following an i.p. injection of 1mg/Kg LPS, the peak mitotic activity occurred on the first day and was essentially normal thereafter. Like SRBC, the mitotic surge stimulated by LPS was accompanied by the development of an hypercalcaemia which was maximal on the first day (11.06 $\pm$ 0.12mg%). This may suggest that a product of B-cell activation is responsible for the development of hypercalcaemia.

The multiplication of cells within the bone marrow is usually seen as a response to the need for new cells in the blood stream or elsewhere. In the embryo and young animal the high rate of proliferation in the bone marrow reflects the large cellular output from this compartment to other tissues of the body. In particular the lymphoid cells are seeded into the spleen and thymus where they undergo further differentiation to B- or T-lymphocytes. Considering the proliferation which was observed in the bone marrow following

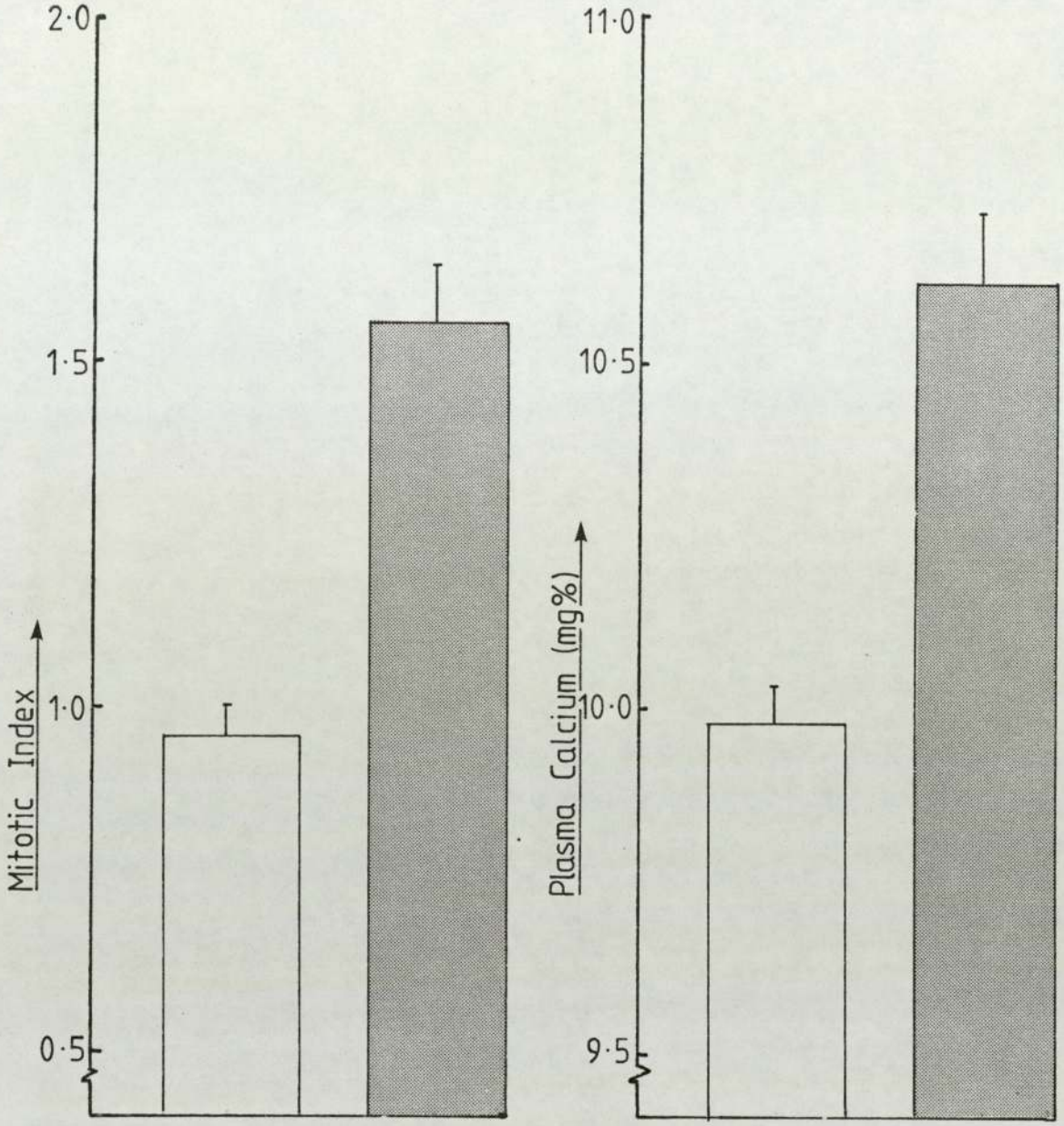


Figure 2.5. The effect of a skin xenograft (shaded bars) on the mitotic index of the bone marrow and plasma calcium. As with other antigenic challenges a proliferative response was observed in the bone marrow ($p < 0.001$) associated with which was a significant hypercalcaemia ($p < 0.001$). Values are means $\pm$ S.E.M. of 8 animals in each case.

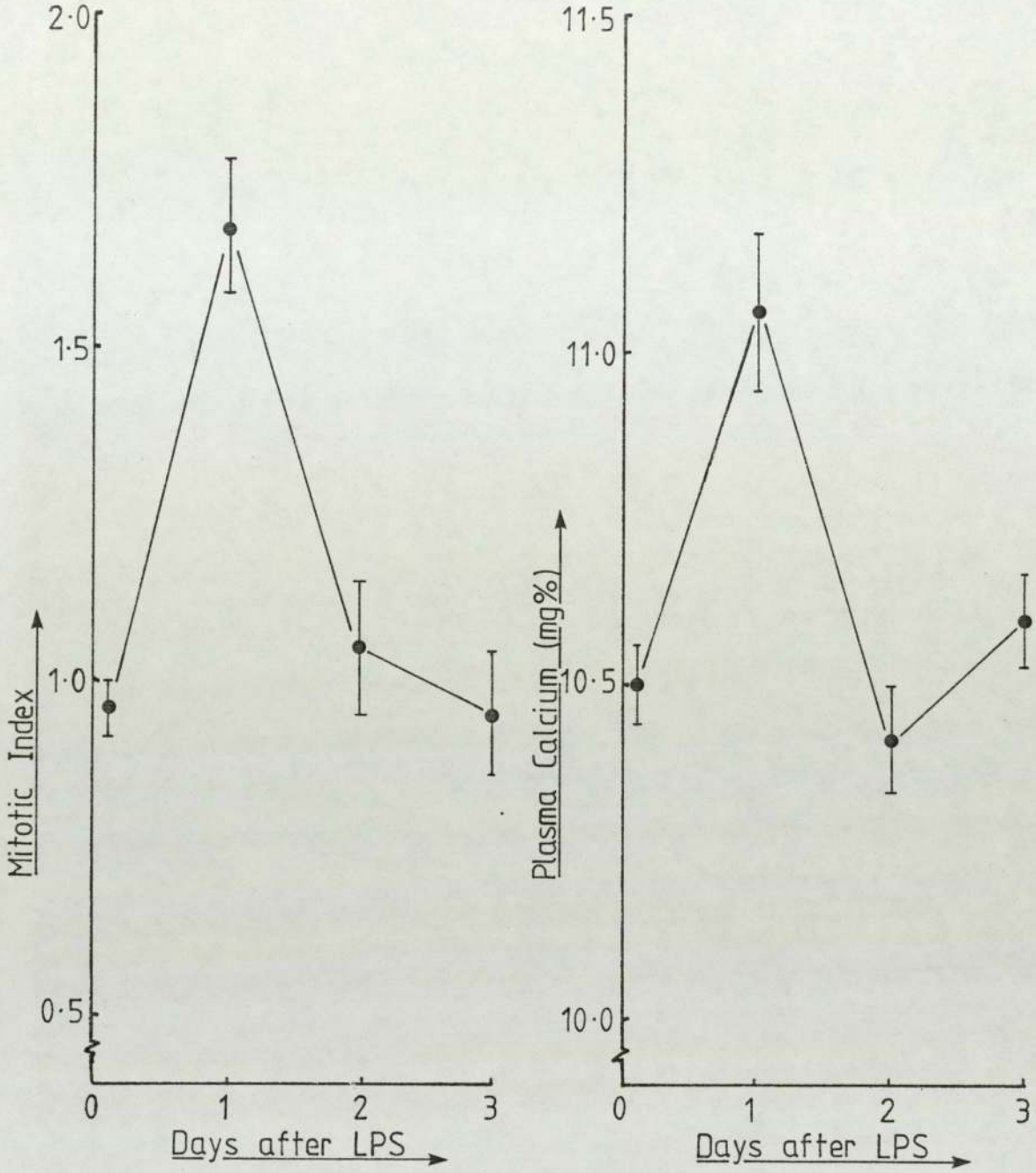


Figure 2.6. The effect of bacterial lipopolysaccharide (LPS) on the bone marrow mitotic index and plasma calcium. The rapid burst of proliferative activity in the bone marrow on the first day after LPS was directly paralleled by plasma calcium concentration. Both mitotic index plasma calcium had returned to essentially normal values by day 2. Values are means ± 1 S.E.M. of 8 animals in each case.

antigenic challenge, it seemed likely that the cells which result from this wave of proliferative activity may likewise leave the bone marrow compartment. It seemed reasonable therefore to expect that the wave of proliferation in the bone marrow might be followed by the appearance of additional cells in the circulation and to test this prediction, total and differential peripheral blood leukocyte counts were measured following antigenic challenge.

The total peripheral leukocyte count was determined using an electronic particle counter (Coulter electronics). Whole blood (heparinised as described previously) was diluted 1.200 with isotonic saline. The erythrocytes were lysed with 6 drops/20ml of the surfactant Zaponin (Coulter Electronics). The sample was mixed immediately after adding the Zaponin and again just before counting. The Zaponin will eventually begin to lyse the leukocytes also but a preliminary experiment (results not shown) indicated that all the erythrocytes were lysed rapidly within 30 secs. A stable leukocyte count was then obtained for up to 60 minutes after addition of the Zaponin, following which the counts gradually declined. It was found convenient to take the leukocyte count 2 minutes after addition of the lytic agent and this procedure was adopted routinely. The count obtained (displayed digitally) was corrected for coincident counts using the tables provided and then for the dilution factor (1/200) imposed. Results were expressed in millions of leukocytes per ml of whole blood.

Using the above procedure, peripheral leukocyte count following challenge with SRBC was found to be significantly elevated by day 4 ($p<0.05$) (fig.2.7). The numbers of peripheral leukocytes remained significantly elevated on day 5 ($p<0.05$) and rose to a peak on day 6 ($p<0.01$). The decline to basal values took place rapidly between days 6 and 7. Differential counts made from

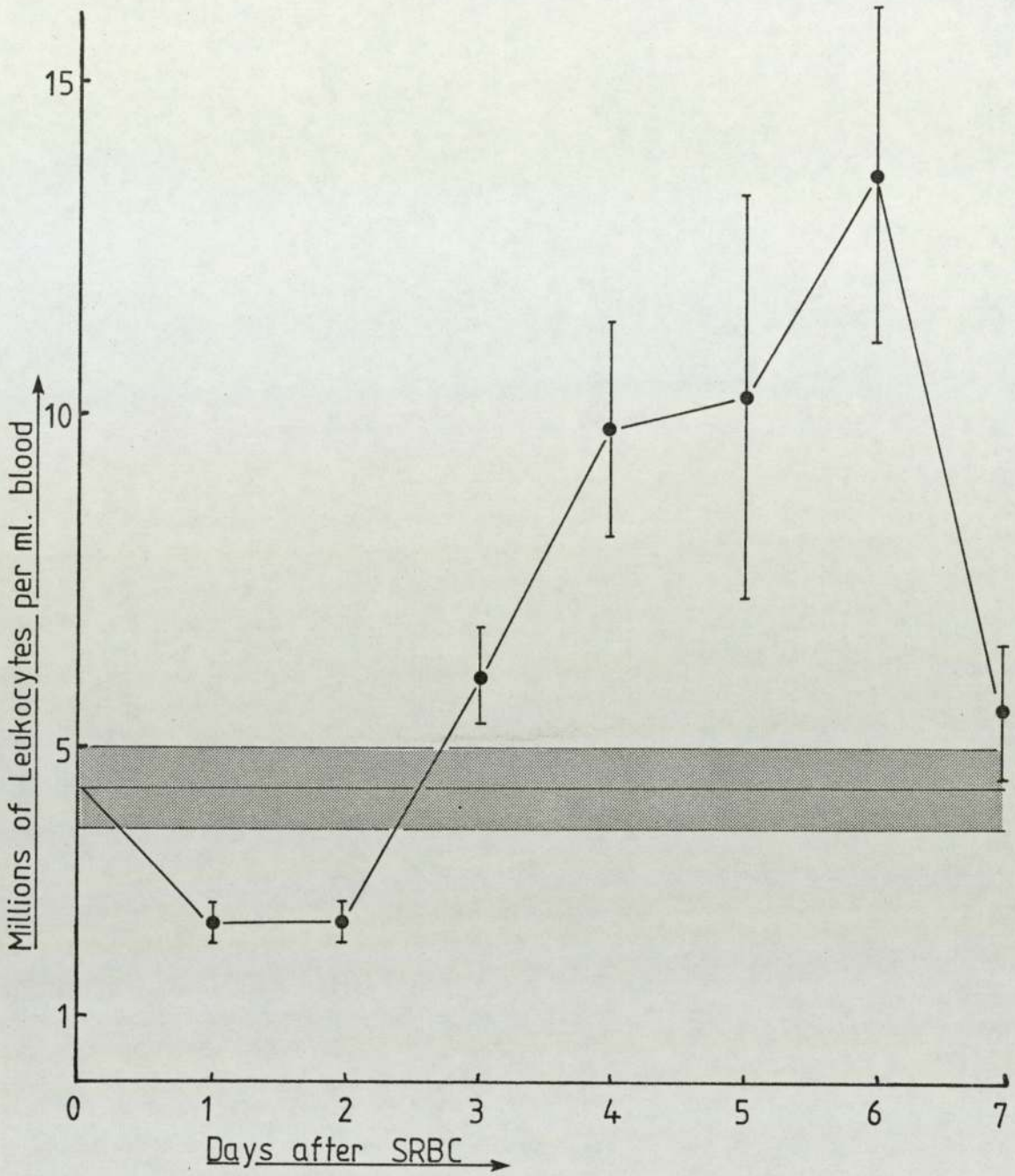


Figure 2.7. The effect of antigen on the total peripheral leukocyte count in rats. Sheep erythrocytes were administered i.p. as a 10% suspension in saline on day 0 at a dose of 1ml. per 200g rat. Whole blood samples were counted on a Coulter counter following dilution and lysis of the erythrocytes. The numbers of peripheral blood leukocytes dropped significantly on the first two days ($p<0.05$). However, a significant leukocytosis had developed by day 4 ($p<0.05$) which persisted on day 5 and rose to a peak on day 6 ($p<0.01$). The return of peripheral blood leukocytes numbers to basal values was rapid so that by day 7 values were not significantly elevated from normal. Values shown are means $\pm$ 1 S.E.M. of between 4 and 18 animals.

Giemsa-May Grünwalds stained blood smears remained unchanged throughout the response to SRBC. These results indicate that since lymphocytes account for 70-80% of white blood cells (WBC) in rat blood, the majority of the new cells were derived from lymphocyte precursors. It is not possible to say with certainty that they were exclusively the progeny of the mitoses seen in the bone marrow or elsewhere one day earlier for they might also have been the result of the extrusion of sequestered mature WBC's from lymphoid reservoirs such as the spleen.

When weighed fresh and cleared of all fat, the relative spleen weight was found to rise significantly following challenge with SRBC (fig.2.8). Thus a progressive splenomegaly developed by day 7 as the WBC count returned to normal. Whether this represented capture and sequestration of the additional circulating WBC's or was the result of splenic proliferation, or both, cannot of course be revealed by these results. Whatever the reason, the 37% increase in relative spleen weight would represent a large increase in absolute cell numbers.

During the response to LPS the total white blood rose by 93% from 4.4 to 8.5 million cells per cubic millimeter. Unlike the response to SRBC, there was a disproportionate increase in granulocytes which accounted for 36% in the LPS-stimulated group compared with less than 16% in normal animals. Some of these increases can be accounted for by changes in cell traffic. However, considering the observation that the bone marrow mitotic index was elevated following LPS some of the cells were almost certainly produced by cell division.

The results obtained have characterized some of the proliferative events of the primary immune response of rats to SRBC and have established that these are associated with the development of an

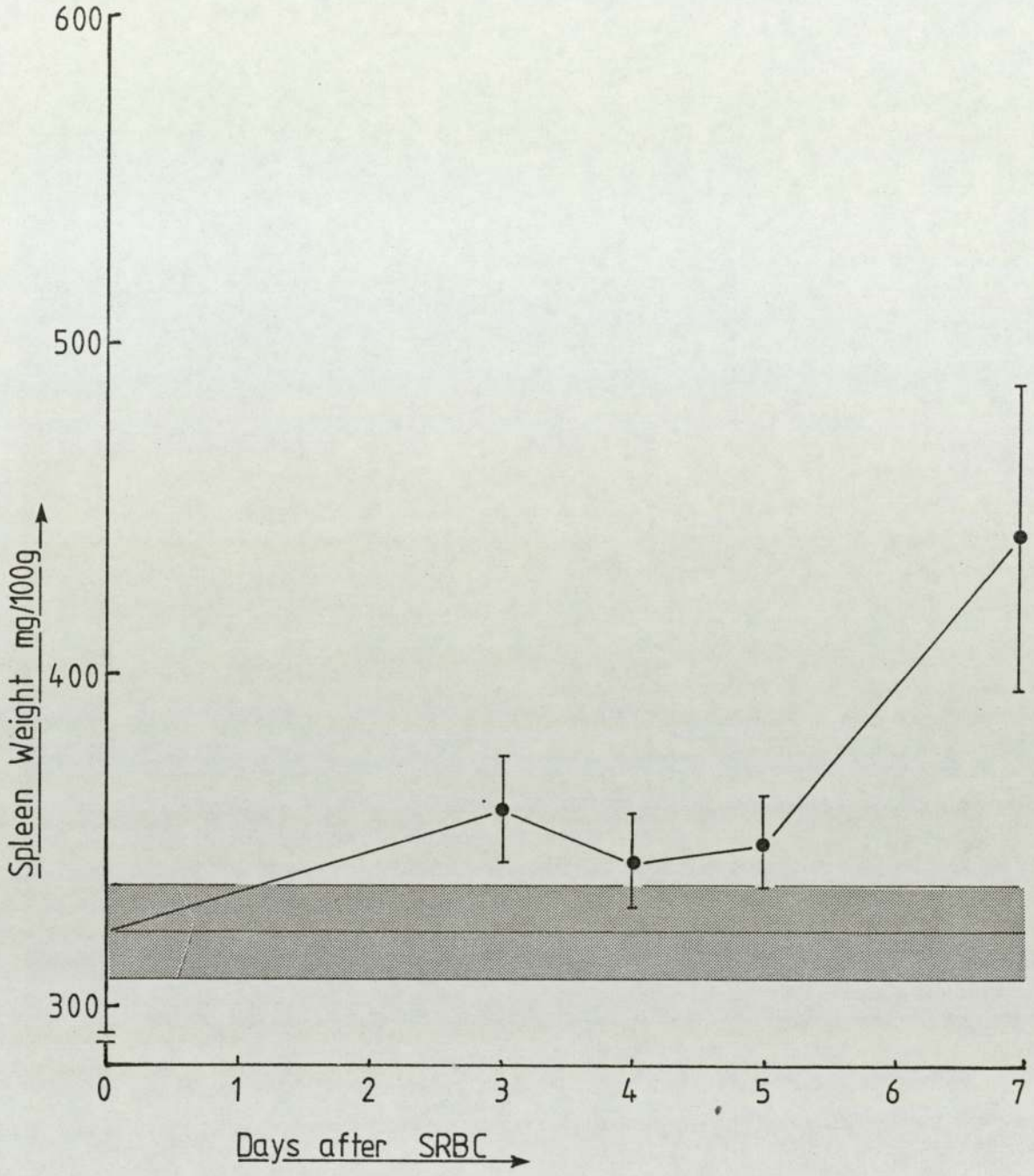


Figure 2.8. The effect of antigen on the relative weights of the spleen. Sheep erythrocytes were injected i.p. on day 0 as a 10% suspension in saline at a dose of 1ml per 200g rat. Spleens were removed from freshly killed animals cleared of adherent fat and weighed. The wet weight is expressed as mg/100g rat. All rats were 200-250g in weight. The relative weight is slightly elevated on days 3,4 and 5 but only reaches statistical significance ($p<0.01$) on day 7. After day 7 values return to normal. Values shown are means $\pm$ 1 S.E.M. of between 4 and 18 animals.

hypercalcaemia in the same manner as in the proliferative events cited earlier (section 1.4). The calcium which appears in the plasma is probably derived from one or more of three sources (section 1.4). Briefly the calcium may be derived from dietary sources or from enhanced bone resorption or may be the result of reduced excretion of the ion. The lack of either a proliferative response or the hypercalcaemia when isologous, rat erythrocytes rather than heterologous sheep erythrocytes were injected suggests that antigenicity is a crucial stimulus for both responses. These results therefore suggest that a product or products of the immune response may be responsible for the observed hypercalcaemia.

No matter which method of antigenic stimulation was used, the hypercalcaemia always occurred concomitantly with the peak proliferative response in the bone marrow. In most cases this was the third day but it was of interest that LPS elicited both responses at one day following administration. LPS is a polyclonal B-cell activator (Andersson et al.,1972; Greaves & Janossy,1972) and probably acted in this manner in the experiments which have been described. Assuming that the hypercalcaemia was stimulated by the same mechanism regardless of antigen then the result obtained with LPS suggests that it may be a product of B-cell activation which mediates the response. The immune response to SRBC demands co-operation from T-cells while LPS can substitute for this helper function in some in vitro responses (Sjöberg, Andersson & Möller, 1972). The T-cell provides a second signal to the B-cell, the first being provided by the antigen, and both signals are thought to be required for activation of B-cell (see section 1.7). An interval of three days in the case of the responses to SRBC may reflect the time taken for the generation of the appropriate signal. This period may also involve the trapping of antigen by macrophages and

the formation of substances from macrophages which non-specifically activate B-cells. In contrast, LPS-stimulated B-cells are activated directly, in the absence of T-cell help, with the consequent rapid wave of proliferation. It is possible therefore that in animals injected with LPS, the co-operative function of the T-cell is bypassed so that B-cell activation and secretion of the putative hypercalcaemic agent occurs more rapidly. This scheme explains the differences between the responses to LPS and SRBC in terms of their differential effects on B-cell activation. However, a hypercalcaemia also developed following a skin graft when heavy T-cell involvement would be expected. Unfortunately no immune response can involve only one type of lymphocyte, since almost any antigen will provoke co-operation from several classes of immunocytes which results in the optimum development of a controlled immune response to that antigen (section 1.7). The cytotoxic action of a sub-population of T-cells (T_C-cells) on graft tissue is assisted by T_H cells (helper T-cells) and does not require any B-cell interaction. However, this represents only one way by which grafts may be rejected. Thus although a skin xenograft elicits a powerful CMI response this does not preclude B-cell involvement.

The increase in plasma calcium concentration which followed challenge by these various antigens clearly represents a deviation from the relatively tight homeostatic control which normally operates. The experiments considered in the next chapter were designed to examine what rôle, if any, the parathyroid gland might play in these events.

2.2 The Rôle of the Parathyroid Glands in the Proliferative Response to Immunological Challenge.

It is apparent that plasma calcium concentration and cell division in bone marrow or thymus are closely linked, since an increase in plasma calcium ~~being~~ is directly paralleled by increased cell proliferation. The changes in plasma calcium which were found to occur following antigenic challenge (section 2.1) were larger than those normally encountered and must therefore represent some derangement in homeostatic control.

There are several possible origins of the calcium which appears in the serum. For example, increased bone resorption, increased absorption of calcium from the diet or decreased calcium excretion would all lead to a positive calcium balance. A prime hormonal candidate for the induction of hypercalcaemia is parathyroid hormone (PTH). Experiments were therefore designed to test the hypothesis that PTH may be implicated in the observed hypercalcaemic phase following immunological challenge.

It should be possible to demonstrate that the hypercalcaemic phase does not occur in the absence of functional parathyroid glands. The parathyroidectomized rat provided a most useful model by which this prediction could be tested.

Rats were lightly anaesthetized with ether and placed back down on an operating table with an etherized pad over the nose to maintain anaesthesia. Ether was found preferable to sodium pentabarbitone (Nembutal) as it is more easily controlled and resulted in far fewer fatalities. One common problem with ether is its tendency to irritate bronchial membranes with consequent mucus production. This sometimes results in arrest of breathing which without action leads to certain death. The most effective way of

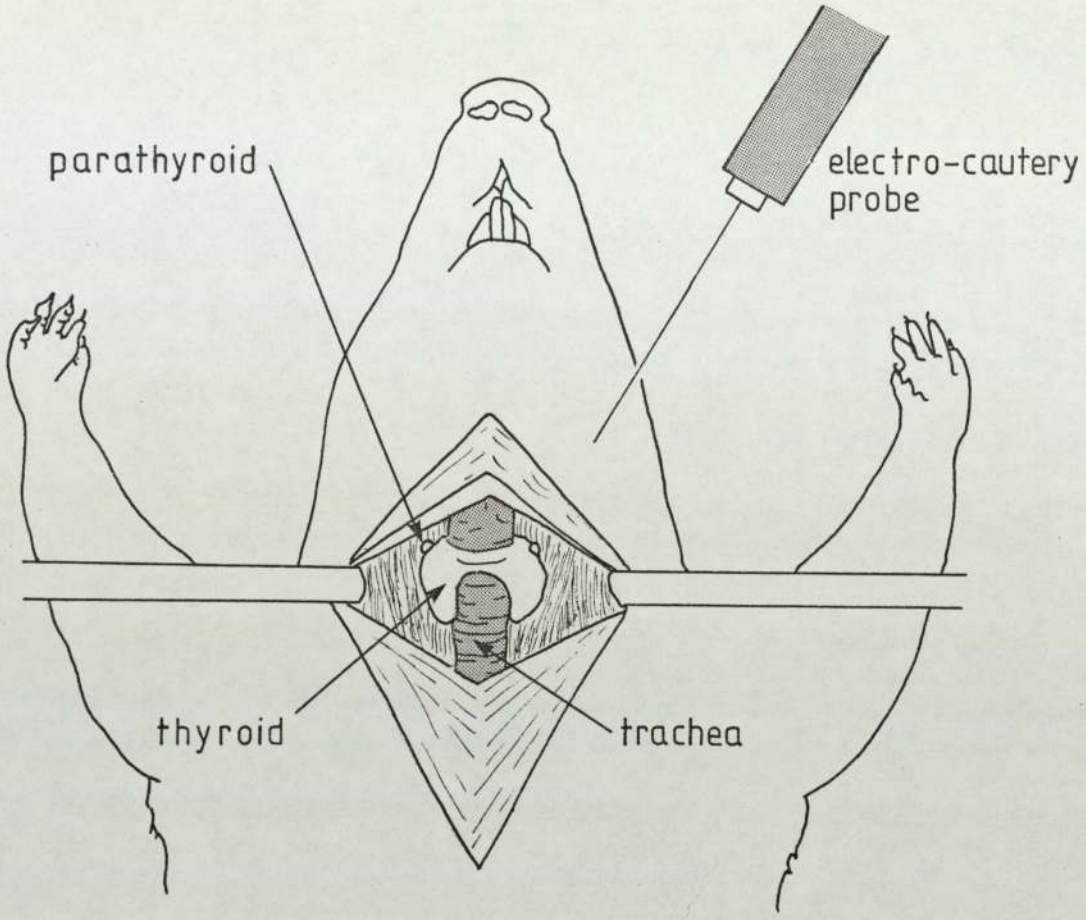


Figure 2.9. The exposure of the thyro-parathyroid complex prior to removal of the parathyroids by electrocautery. After making the appropriate in cision and retracting, the thyroid can be cleared of any obscuring tissue so that clear access can be gained to the parathyroids. The rat was placed on the neutral electrode of the electrocautery unit while the probe is live. When in position, the current was switched on by a foot switch which resulted in destruction of the area in the immediate vicinity of the probe tips where current density is greatest. After the cautery, which takes just a few seconds, the muscles were pinched together to promote healing and the wound closed with clips. (After Munson & Hirsch, 1966).

resuscitating the animal was found to be with a length of rubber tubing placed over the nose of the rat and through which the lungs could then be ventilated by short blows. Using this method, breathing could be quickly restarted and fatalities were reduced essentially to zero. When suitably secured on the table the throat region was shaved and swabbed with antiseptic solution (lyseptol; BDH). All instruments were cleaned and soaked in lyseptol solution (BDH) prior to use and returned there between use. A 2-3cm median saggital incision was made through the skin in the neck region. Using fine scissors the connective tissue between the two submaxillary salivary glands were cut thus exposing the sterno-hyoid muscle which overlies the trachea and thyro-parathyroid complex. Access to the trachea was obtained by making a lateral longitudinal incision into the muscle with the closed points of a pair of scissors. Opening the points of the scissors then resulted in the muscle parting along its length. The split muscle was retracted laterally thus exposing the trachea and thyro-parathyroid complex. Connective tissue and the two small omohyoid muscles were cleared from the adjacent thyroid tissue so that the parathyroid glands, which lie one in each lobe of the thyroid towards the upper pole (fig.2.9), could be seen. Each parathyroid gland was about the size of a pinhead and was visible naked-eye as a bluish-grey plaque on the thyroid surface. The parathyroids were removed by electrocautery (Munson & Hirsch,1966) using a magnifying loop to aid the operation. Care was taken to avoid damaging the recurrent laryngeal nerves, which run either side of the trachea, (fig.2.9) as this results in severe imparement of the normal breathing rhythm. When the operation was completed the sterno-hyoid muscle was lightly pinched together to promote quick healing and the wound closed with wound clips. Parathyroidectomized (PTX) animals had both parathyroids removed

by this method while sham PTX were cauterized on the lower pole of the thyroid away from the parathyroid. This mimics the slight damage of the thyroid inevitably incurred during the PTX operation and also causes an equivalent release of thyrocalcitonin and resultant short-lived hypocalcaemic phase (Munson & Hirsch,1966). The loss of thyroid tissue and hence reduced thyroxine secretion is quickly compensated for by enhanced secretion from the remaining tissue under the influence of thyroid stimulating hormone. After the operation all animals were placed on normal diet and water ad libitum.

Following parathyroidectomy or the sham operation all animals enter a transient hypocalcaemic phase (fig.2.10) caused by release of thyrocalcitonin from thyroid tissue damaged during the operative procedures (Munson & Hirsch,1966). Sham parathyroidectomized rats showed a rapid recovery but often had plasma calcium concentrations slightly lower than intact animals. In contrast, the aparathyroid animals showed no recovery but rather, developed a persistent hypocalcaemia which lasted for several weeks (fig.2.10). This result contrasts with those obtained with aparathyroid Sprague-Dawley rats which re-establish normocalcaemia within 4 days if allowed a normal diet ad libitum (Perris & Whitfield,1971). The development of hypocalcaemia in aparathyroid animals is caused by the absence of PTH resulting in greatly reduced bone resorption and increased calcium loss in the urine (see section 1.4). It might be expected that hypocalcaemic conditions would favour 1,25 DHCC production leading to increased amounts of CaBP and hence greater extraction of calcium from dietary sources. If aparathyroid rats were placed on low calcium diet (less than 10mM compared with 150mM in normal diet) or starved overnight, the calcium concentration of the plasma did drop by a further 1mg% (to around 5.5mg%) which probably indicates

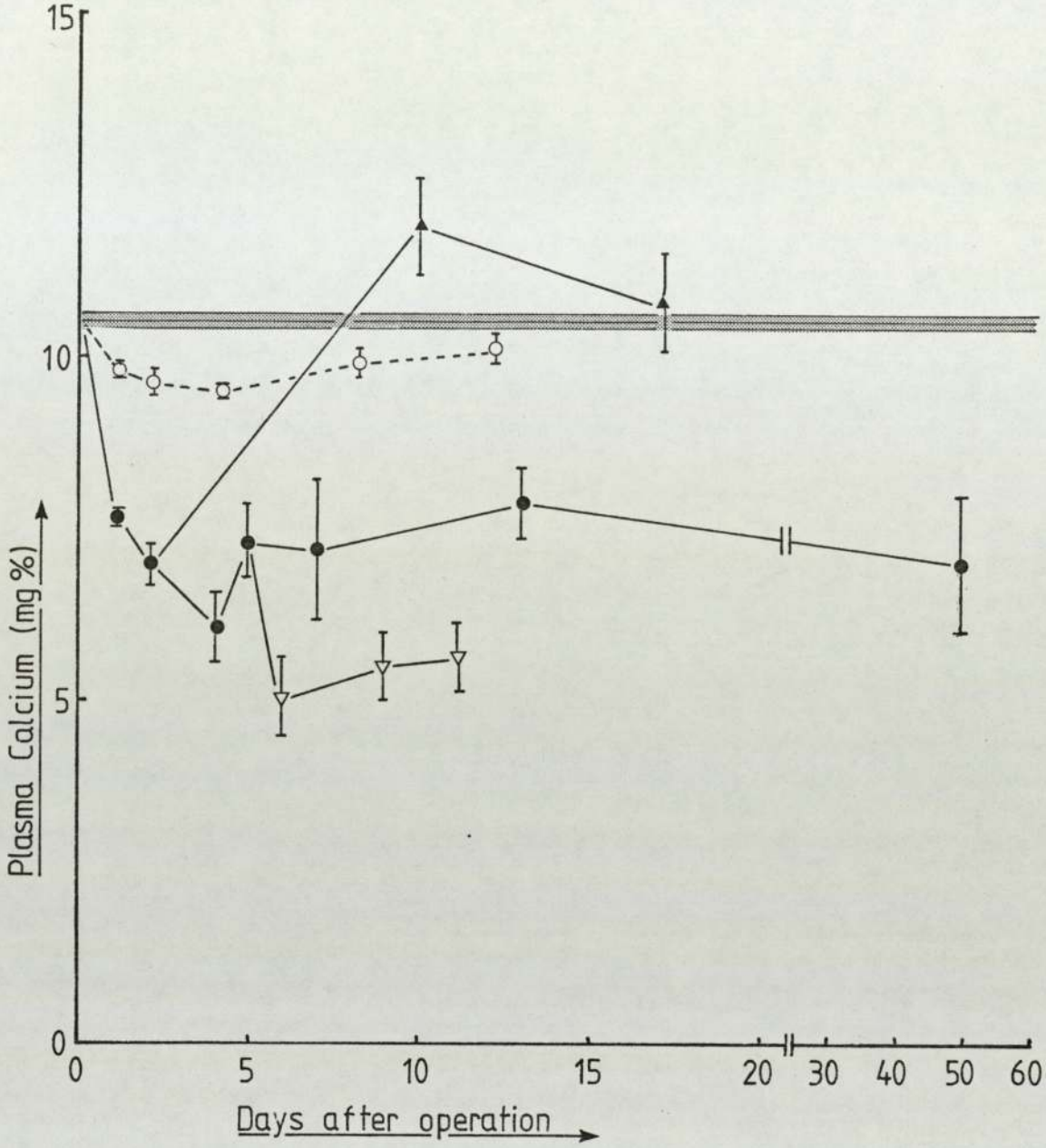


Figure 2.10. The effect of parathyroidectomy on the plasma calcium concentration. The horizontal line and shaded area shows the mean ± 1 S.E.M. for normal calcium concentration and illustrates how little variation there is in the intact animal. Animals parathyroidectomized (PTX) on day 0 and fed on normal diet (●) rapidly become hypocalcaemic. Although some recovery takes place, the animals remain markedly hypocalcaemic with plasma calcium concentrations around 7mg% for several weeks. (continued over).

Figure 2.10. (continued). If these animals were placed on low calcium diet (▽) a further drop in plasma calcium occurred, stabilizing around 5.5mg%. However, replacement with high calcium diet (▲) resulted in rapid recovery to normocalcaemia though some overshoot occurred and less precise homeostatic control was evident by greater variation in plasma calcium concentration. Sham PTX (O) also results in the development of a minor hypocalcaemic phase but recovery occurs within the first week to values only just below normal.

that dietary calcium compensates in part for PTX -induced hypocalcaemia. The difference between Wistar and Sprague-Dawley strains may be due to different laboratory diets or may reflect reduced extraction of calcium from the diet by Wistars. In support of this last point it was noticed that the inclusion of calcium gluconate in the drinking water of aparathyroid Wistar rats also failed to induce normocalcaemia. This treatment has previously been shown to be effective for Sprague Dawley rats (Perris & Whitfield,1971). To obtain normocalcaemic aparathyroid rats a high calcium/low phosphate diet was provided (300mM) (Robinson & MacIntyre,1967) following which parathyroidectomized animals became normocalcaemic within 14 days (fig.2.10).

Sprague-Dawley rats kept on low calcium diet for one week have only 75% survival rate (Perris & Whitfield,1971). It would be undesirable to perform tests of immunological competence (chapter 2.3) on animals in such poor health as this may give spurious results. Also some of the experiments which were performed required the animal to spend a protracted period in an hypocalcaemic state which would have been impossible if a low calcium diet was fed because of excessive mortality. It was decided therefore to use PTX animals on normal diet for most of the experiments. This regimen provided animals in good health but with a considerable degree of hypocalcaemia which remained constant for several weeks (fig.2.10) and minimized the possibility of stress caused by severe hypocalcaemia.

Failed PTX operations were usually detected at the time of sacrifice. With the PTX -normocalcaemic rats a small tail vein sample was obtained for the purpose of calcium titration 3 days post-operatively and successful PTX rats (less than 8mg%) placed on high calcium diet. Parathyroidectomy was successful in 95% of operations.

Groups of rats were operated on as described above and replaced on normal food and tap water ad libitum. Seven days post operatively they were immunized with SRBC in saline (preparation described in section 2.1) or received a control injection of saline alone. The animals were sacrificed 3 days following immunization and the bone marrow mitotic index and plasma calcium assessed as described previously (section 2.1). The results (figs.2.11; 2.12) showed that while in both normal intact and sham PTX rats there was the usual elevation of proliferative activity, this was ablated by PTX. It is interesting that both the control and immunized values of MI for sham PTX are higher than their counterparts in intact animals. This may reflect potentiation of the response by small quantities of corticosteroids released following the stress of operation. However, the main feature of the graph is the complete obliteration of any proliferative increment in immunized PTX animals compared with unimmunized PTX controls. In parallel with this result a hypercalcaemia fails to develop in immunized PTX animals (fig.2.12). This is in sharp contrast to sham PTX and intact animals, both of which show significant elevation of plasma calcium in the immunized groups (fig.2.12). These results imply that in some way the presence of an intact parathyroid gland is required for the proliferative response of the bone-marrow to antigen. The concomitant absence of hypercalcaemia strongly suggests that the elevated bone marrow MI was mediated by plasma calcium levels.

From these results it was impossible to say whether the lack of proliferative response was due to the absence of an hypercalcaemic episode or simply because the hypocalcaemic environment was unsuitable for the expression of such a response. The use of aparathyroid animals rendered normocalcaemic by diet as described above acted as a control experiment for this possibility. The results

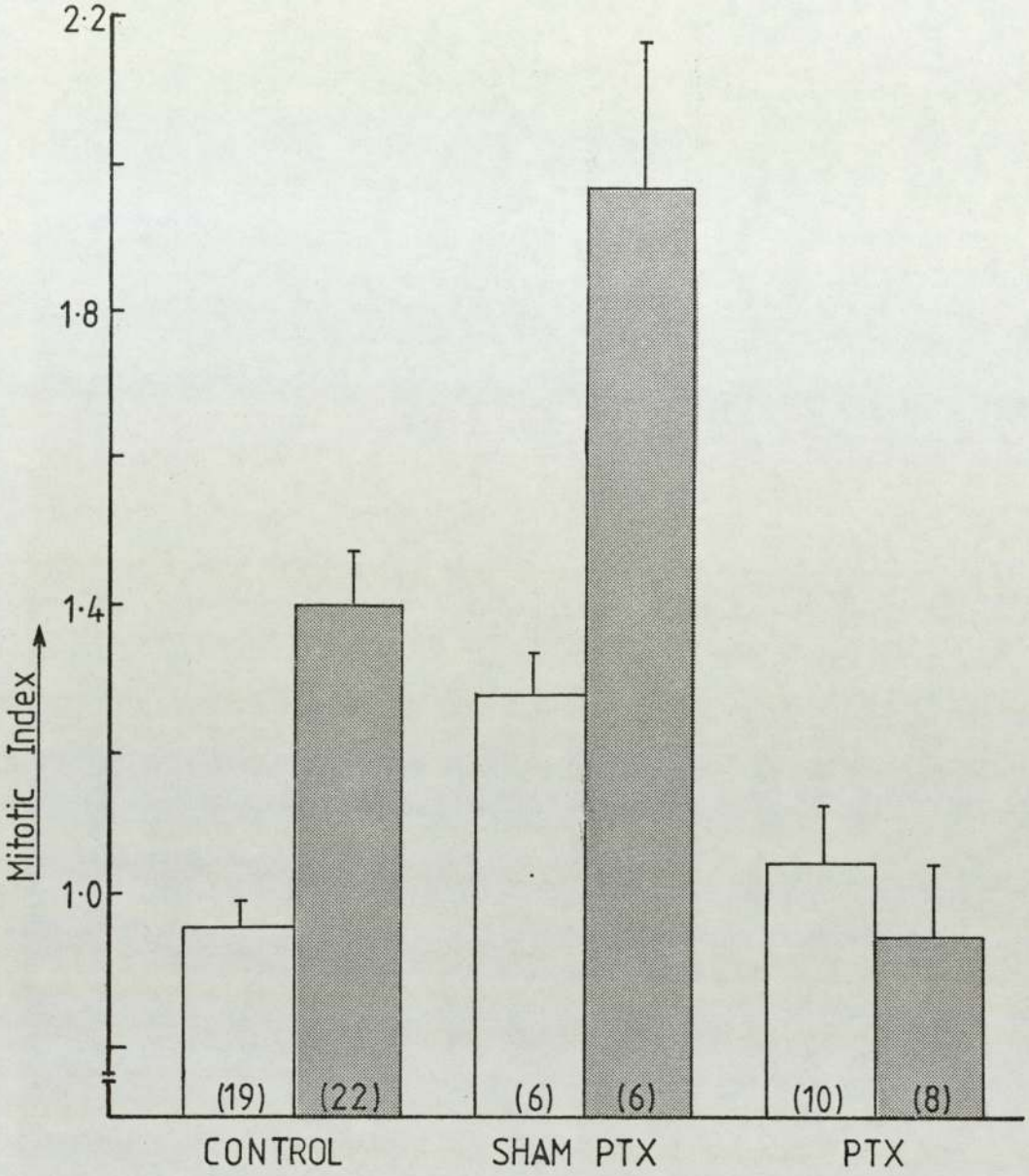


Figure 2.11. The effect of antigen on bone marrow mitotic index in normal, sham operated and PTX rats. Results were obtained on the third day following an i.p. injection of 10% SRBC in saline (shaded bars) and are compared to saline injected controls (open bars). In both the control (intact) animals and sham PTX group a highly significant ($p<0.001$) elevation of mitotic activity occurred. In contrast, no such increment was found in parathyroidectomized (PTX) rats and indeed the mean of the injected PTX rats was lower than the PTX control group though this was not statistically significant. The sham operation appears to have had a potentiating effect since sham PTX animals had particularly high values for both antigen challenged and unchallenged groups. The number of animals used is shown in parentheses. Values are means $\pm$ 1 S.E.M.

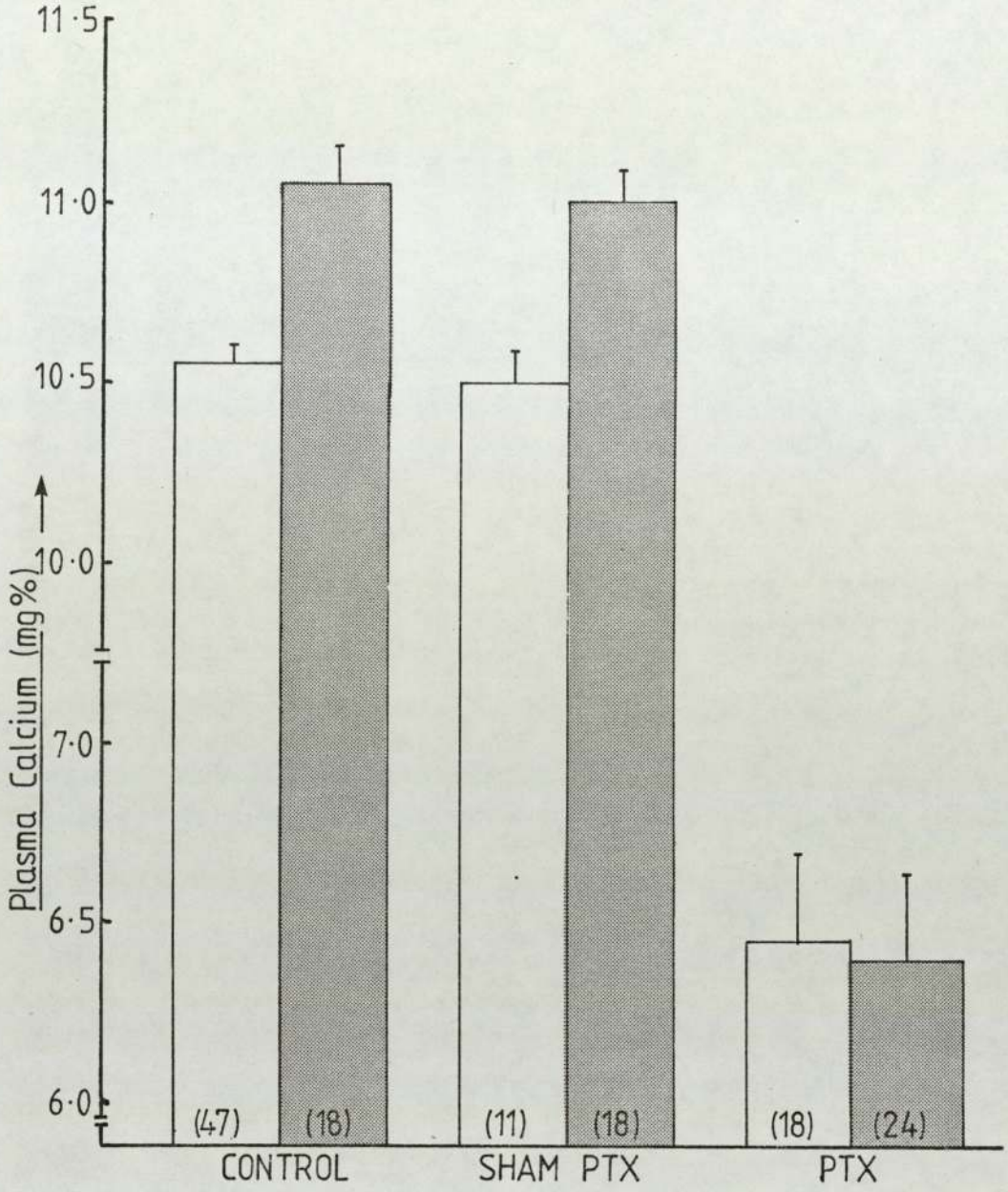


Figure 2.12. The effect of antigen on plasma calcium concentration in normal, sham operated and PTX rats. The values are means ± 1 S.E.M. obtained three days after challenge with 10% SRBC (shaded columns) or saline (open columns). In both intact and sham PTX groups a significant hypercalcaemia developed ($p<0.001$ for intact, $p<0.02$ for sham PTX). The plasma calcium of the PTX animals was just above 6mg% following the operation and was not affected by antigenic challenge. These results directly parallel the effect of antigenic challenge on the bone marrow mitotic activity (fig.2.11) and thus serve to confirm that aparathyroid hypocalcaemic rats are unresponsive to antigenic challenge in either the production of elevated bone marrow mitotic index or elevated plasma calcium.

(fig.2.13) show that rats without parathyroids but with normal plasma calcium levels were still unable to mount a proliferative response in the bone marrow following antigenic challenge. Furthermore there was no incremental increase in plasma calcium. The distribution of plasma calcium concentration around the mean is greater than in normal animals and probably reflects the reduced homeostatic control of these aparathyroid rats.

This result demonstrates that an intact parathyroid gland is required for the development of antigen induced hypercalcaemia and increased bone marrow proliferation. The lack of a proliferative response in PTX hypocalcaemic animals cannot be ascribed to the unsuitability of the low calcium environment for its proper expression since in normocalcaemic conditions no incremental increase of mitotic activity is found in immunized aparathyroid animals. This result thus suggests that the parathyroids are somehow responsible either directly or indirectly, for driving the enhanced proliferation by a mechanism mediated by the hypercalcaemia.

There are other possible sources of calcium in the antigen--induced hypercalcaemic response. As outlined in section 1.4, the three major sites at which plasma calcium is controlled are the bone, gut and kidney (fig.1.8). An experiment was undertaken to see if the hypercalcaemia which occurred after antigenic challenge could be prevented not only by PTX (see figs.2.12., 2.13.) but by prevention of influx of calcium from dietary sources or retention via the kidney.

Bacterial lipopolysaccharide (LPS) was chosen as the challenging agent because it had been shown to induce a rapid hypercalcaemia in 24 hours (fig.2.6). This allowed experimental manipulations on a short term basis which would have otherwise been impossible. To investigate the intestinal route as a possible source of calcium,

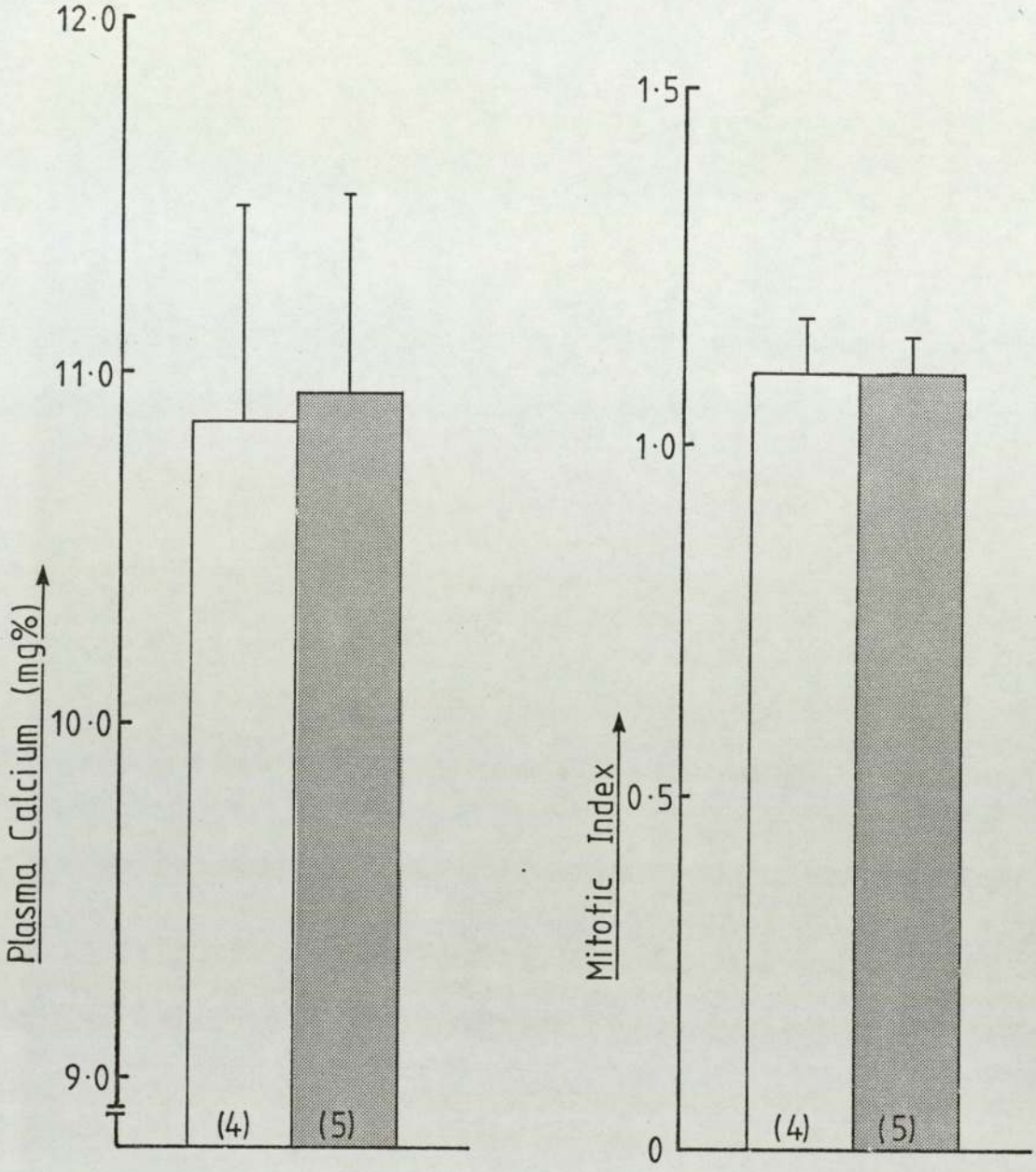


Figure 2.13. The lack of effect of antigenic challenge on plasma calcium and mitotic index in aparathyroid animals fed on high calcium/low phosphate diet to restore normocalcaemia. All the animals represented in the data were shown to be successfully parathyroidectomized by measuring the plasma calcium prior to placing on high calcium diet. Neither plasma calcium nor the mitotic index was elevated three days following an i.p. injection of 10% SRBC (shaded bars) when compared to saline-injected controls open bars). This experiment eliminates the possibility that a low calcium environment within an aparathyroid rat is alone responsible for the lack of response of parathyroidectomized animals to antigenic challenge and suggests that the hypercalcaemia observed in normal animals is mediated by the parathyroid glands.

rats were starved for 24 hours prior to an i.p. injection of 50 µg/100 LPS (*E.coli*) in saline. The plasma calcium concentration was measured, as described earlier, 24 hours after the challenge. Pre-starving in this manner was intended to empty the intestine and therefore remove any possible source of calcium. Thus any incremental increase in plasma calcium after challenge with LPS following starvation could not be the result of sequestration from dietary sources.

The result (fig.2.14.) shows that while a significant increase in plasma calcium ($p<0.05$) occurred in rats fed normally following LPS, starved rats showed no increase in plasma calcium. This suggests that the intestine may act as a source of calcium during development of hypercalcaemia following antigenic challenge.

To study the possible rôle of the kidney a bilateral nephrectomy (Nx) was performed by ligation. Since this operation prevented the maintainance of plasma ionic balance, it was necessary to substitute 0.9% saline for drinking water. The rats were challenged with LPS (50µg/100g) in saline immediately following the operation and the plasma calcium concentration measured 24 hours later. The result showed that Nx also prevented the hypercalcaemic phase. However the operation alone clearly had an adverse effect on the concentration of plasma calcium since the Nx rats were hypocalcaemic when compared to intact normal animals and exhibited a wide variation in plasma calcium concentration. There is insufficient data to be conclusive, but it would appear that Nx prevented the development of hypercalcaemia following LPS.

Taken together with previous observations these results indicate that while intact parathyroid glands are certainly required for antigen-induced hypercalcaemia to occur, it is also possible that additions of calcium from dietary and/or renal sources may be involved.

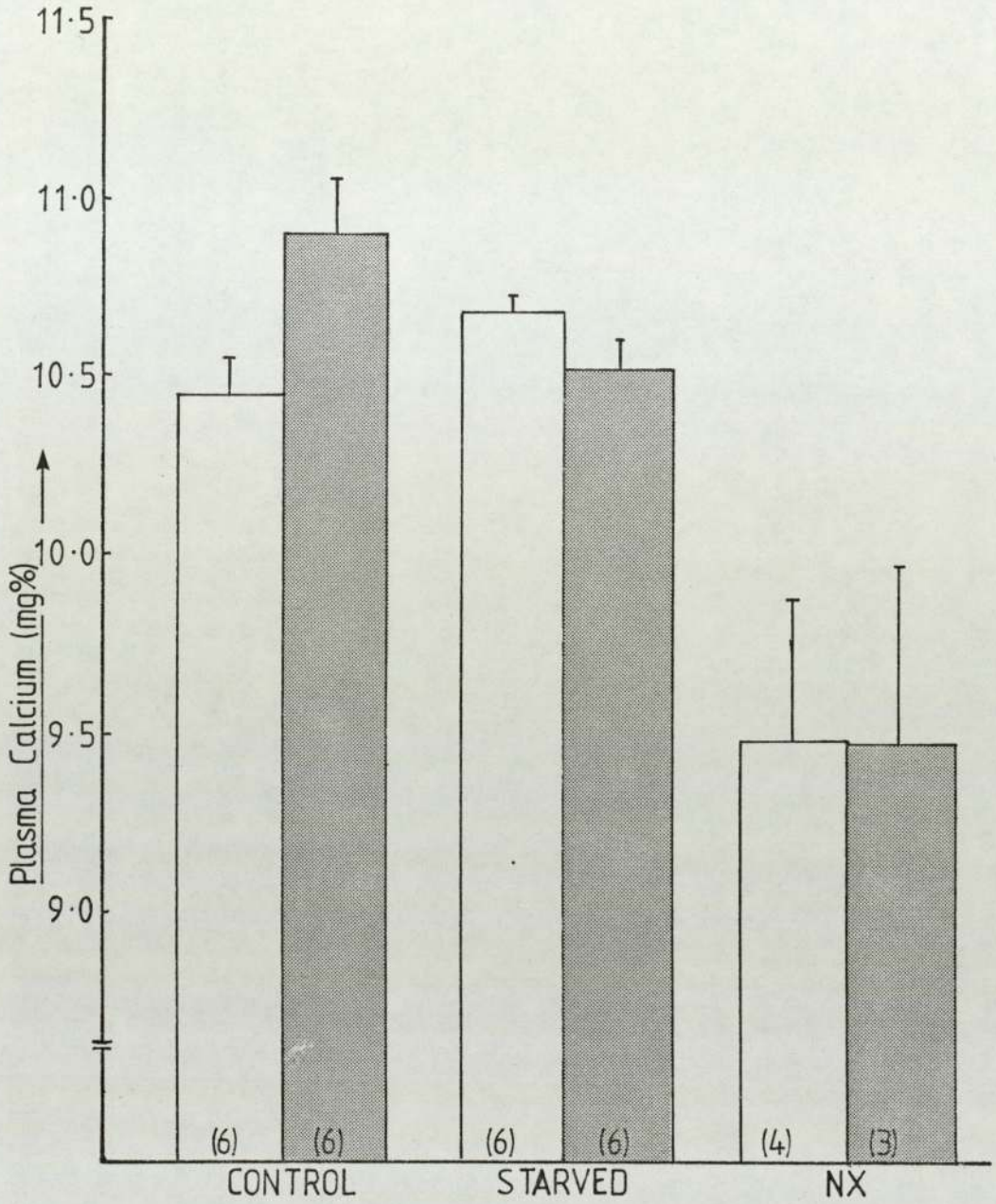


Figure 2.14. The effect of bacterial lipopolysaccharide (LPS) on the plasma calcium concentration in normal, starved and nephrectomized rats. Plasma samples were obtained 24 hours after a single i.p. injection of LPS at a dose of 50µg/100g in saline. A significant hypercalcaemia developed in control animals following LPS ($p<0.05$). However, in rats pre-starved for 24 hours or challenged immediately following bilateral nephrectomy (Nx) no response had occurred one day later. Values are means ± 1 S.E.M. of 3-6 rats as indicated in parentheses.

So far, only the proliferative activity of the bone marrow has been considered. The effect of a proliferative response in the bone marrow following antigenic challenge on the final immune response is difficult to predict. Many of the cells undergoing division are primitive cells which do not mature into specific plasma cells during the primary response. Thus in the primary immune response when the numbers of antibody producing cells in the spleen reaches maximum there are only a tiny number in the bone marrow (see section 2.3). The bone marrow may however play a rôle in the secondary immune response (Benner & van Oudenaren, 1975; Hill, 1976) when considerable number of antibody-producing cells are located there. The splenic lymphocytes have a much clearer rôle in the primary immune response when the spleen becomes a major site of differentiation of virgin B-cells to plasma cells. The numbers of antibody-forming cells in the spleen is accurately reflected by the rising antibody concentration in the primary immune response (section 2.3). It was thus decided to measure the proliferative response in splenic lymphocytes in vivo following challenge with SRBC and to assess whether such responses were affected by parathyroidectomy.

Autoradiography was used as a particularly sensitive method of assaying for increased numbers of splenocytes entering DNA synthesis. Techniques involving the uptake of iodonated deoxyuridine (^{125}IUDR) or tritiated thymidine (^{3}HTdR) and subsequent gamma or liquid scintillation counting were found to be insufficiently sensitive for this purpose (appendix 1).

The technique of autoradiography involves the culture of cells with a radioactive precursor molecule, usually labelled with tritium (^{3}H) which is most suitable because of the low-energy of the β-radiation emitted. A histological specimen of the

radioactive sample is then prepared and covered by photographic emulsion which became exposed in those areas subjected to irradiation. The short distance travelled by the low-energy β-particles of tritium (1-2μm) increases the resolution of the method considerably. Tritiated thymidine (^{3}HTdR) will, if included in culture medium, be incorporated only into the DNA of those cells which are in the S phase, so that these cells can be distinguished from those in other phases of the cell cycle.

The method adopted (Pelc,1956; Nossal,1963) was to remove spleens from rats on the required day (usually day 3) following immunization and place immediately in cold (4°C) medium 199 (Flow) buffered with 20mM hepes and supplemented with 10% foetal calf serum (FCS)(Flow). Although calf serum was probably not necessary in these experiments it was included partly for consistency with other experiments and partly because the concentration of FCS affects the viability assessment with trypan blue (see section 2.3). When cleared of all adherent tissue, the spleen was removed to fresh medium (also at 4°C) and minced into fine pieces using curved scissors. The suspension of cells and small particles of spleen were transferred aseptically to a sterile tube and allowed to settle at unit gravity for one minute at 4°C. The suspension remaining after this time consisted of splenic lymphocytes and macrophages heavily contaminated with erythrocytes. Though these contaminants can be removed (section 2.3) it was found unnecessary for autoradiography. It was felt that the less manipulations performed, the less would be the likelihood of introducing artefacts e.g. preferential lysis of cells in certain stages of the cycle which could lead to an artificial relative elevation of the number of cells in another phase). The splenocytes were washed once and adjusted to approximately 10^{7} cells/ml. in one ml. cultures in

sterile round bottom plastic culture tubes (Falcon Plastics Ltd). Tritiated thymidine was added at the concentration of 5μCi/ml of ^{3}H-methyl thymidine (SA = 21 Ci/mM) and the incubation allowed to proceed for 3 hours at 37°C. At 3 hours the uptake of thymidine into these unstimulated cells was found to have reached a plateau which persisted for at least one day under culture conditions described with little drop in viability (85-90%) (fig.2.15). The cultures were stopped by addition of an equal volume of 10% formaldehyde in saline at pH 7.2 and fixation was allowed to proceed for 30 minutes. The cell suspension was then centrifuged (400 x g) and resuspended in a small amount of double distilled water. One drop of this suspension was spread with one drop of FCS on a slide previously cleaned in chromic acid, thoroughly rinsed and subbed with a solution containing 0.5% gelatine and 0.05% chrome alum. The clean, subbed slides were prepared in bulk prior to use by dipping cleaned slides in gelatine/chrome alum, drying in an oven at 90°C and storing in a dry atmosphere separated from each other by paper tissue. The smear of cells with serum was dried in a current of warm air. When dry the slide was given two 5 minute washes in a 10mM thymidine solution at 4°C to replace any unbound radioactive thymidine with non-radioactive thymidine. This was followed by 2 minutes in cold distilled water. Finally the slide was air dried as before when it was ready for application of the autoradiographic film. The samples can be stored dry at this stage for some time. It was found convenient to store overnight in a dry atmosphere at 4°C. The washing procedure helps to reduce to negligible amounts the contaminating tritiated thymidine in the sample which has not been incorporated into DNA. The final result is an autoradiograph with a very low 'background' count.

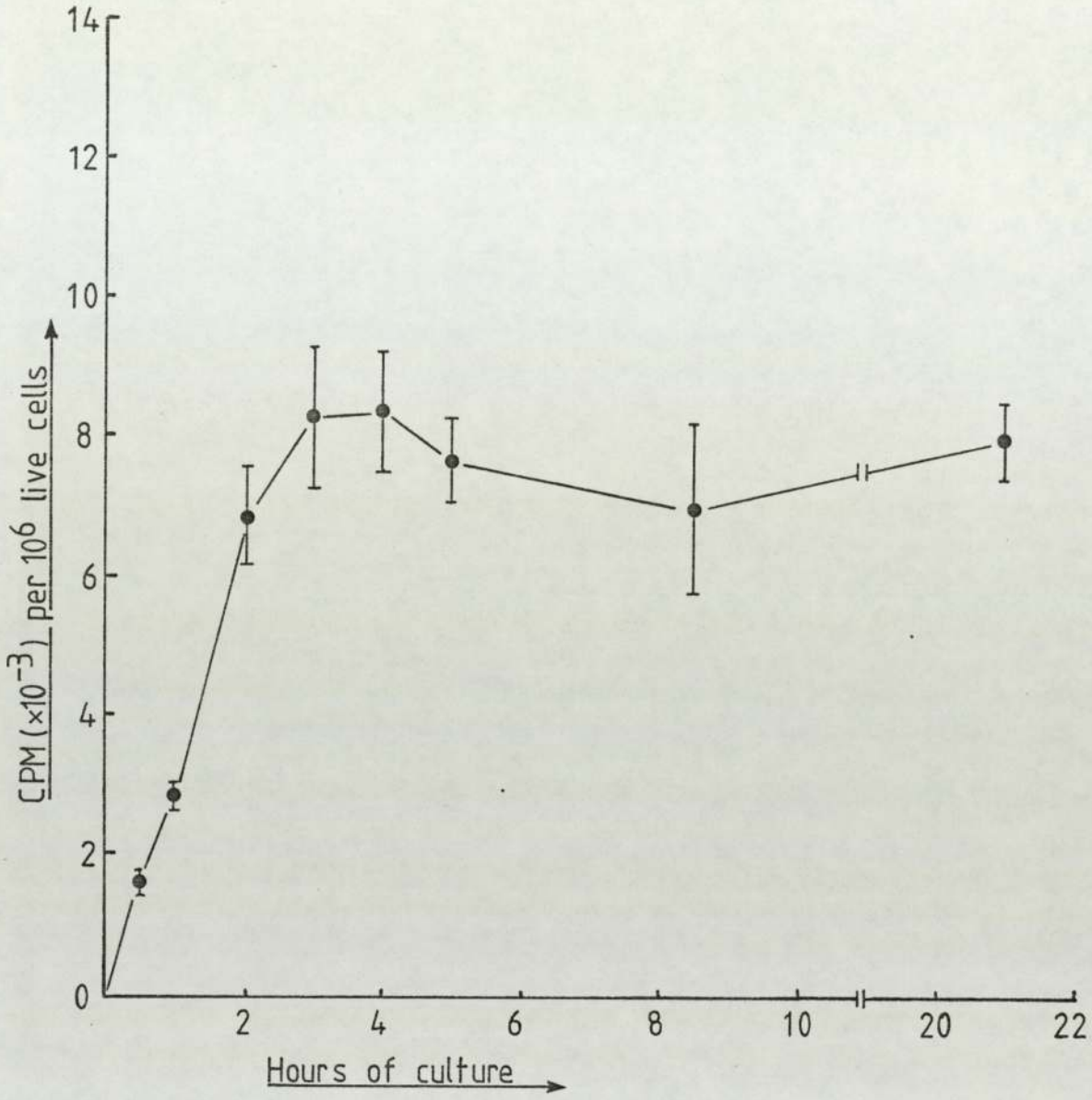


Figure 2.15. The uptake of tritiated thymidine into bone marrow cells in vitro. Bone marrow cells were suspended in medium 199 suplemented with 20% foetal calf serum and buffered with 20mM Hepes at a concentration of 10^7/ml in the presence of 5μg/ml of tritiated thymidine. The cultures were harvested and prepared for liquid scintillation counting as described in appendix 1. At first the incorporation proceded rapidly but reached a maximum value between 2-3 hours and did not diminish for several hours thereafter. Viability was 85-90% throughout as assessed by trypan blue exclusion. Values represent mean ± 1 S.E.M. of six duplicate cultures from separate animals.

The autoradiographic film used was AR 10 (Kodak) which is supplied attached to glass plates. All manipulations with the film were performed in a darkroom under dark red safelight (Kodak No.1) containing a 15W bulb and positioned 1.5m from the working area. If the smears had previously been stored dry, they were soaked in clean distilled water for 10 minutes prior to fixing the film. The film was prepared by cutting pieces from the plate and laying sensitive-side down in a shallow dish of clean distilled water (the sensitive surface is away from the glass plate). The humidity of the dark room was maintained at about 40% by boiling a beaker of water on a hot-plate. This reduced the tendency for static electricity to build up as the film was peeled off the glass plate leading to small sparks which cause heavily grained tracks running accross the final autoradiogram and which are undesirable because of the amount of slide they obscure (Nossal,1963). The humidity was not allowed to rise too high as this caused the film to become 'tacky' and difficult to handle. After two minutes the film was picked off the surface of the water from underneath by the sample slide and allowed to drain after which the film was dried in a flow of cool air. The slides were exposed to the radioactivity of the labelled cells for 4 days at 4°C in a sealed light-tight box containing a little silica gel.

After the required exposure the slides were developed (to visualize the grain of silver) and stained (to visualize the cells). All development and staining procedures were performed with cooled reagents (about 15°C). This prevented the film from swelling to a greater extent than when it was first applied to the sample and thus minimizes the possibility of film slip (Nossal,1963). If extensive areas of the film slips the relationship of grains to cell is lost and the autoradiogram is useless. The slides were first developed

for 4 minutes in cooled Kodak 19b developer made up according to the maker's instructions. After development the slides were rinsed for one minute in distilled water and then transferred to fixative (Kodafix) for 2 minutes. The slides were then rinsed thoroughly (5 minutes) in running tapwater.

Following development the grains are visible over the cells which incorporated isotope during the culture period and it was then necessary to visualize all the cells with sufficient clarity to be able to produce accurate counts of the number of labelled cells in the population. The staining was thus performed through the autoradiographic film and was therefore not of the same clarity expected from histological work. The stain used was Giemsa into which the slides were placed for 15 minutes followed by two washes (1½ minutes each) in clean distilled water. After draining and drying in a stream of cool air, the slides were counted under oil immersion for the percentage of labelled cells. A total of at least 2000 cells were counted by two independent observers and the slides were coded so that the identity was unknown to the observer thus preventing bias.

Using the techniques described above, autoradiograms of bone marrow and spleen were prepared from both immunized and control animals on day 3 following immunization. The effects of parathyroidectomy and sham parathyroidectomy were investigated.

The effect of immunizing rats with 1ml/100g rat of 10% SRBC in saline on incorporation of ^{3}HTdR into bone marrow cells is shown in figure 2.16. Both sham PTX and intact animals showed a significant ($p<0.05$) increase in the percentage of cells which incorporated tritiated thymidine (^{3}HTdR). These results confirm that the bone marrow in the normal rat exhibits a proliferative response to a primary immunological challenge. In contrast to sham PTX and

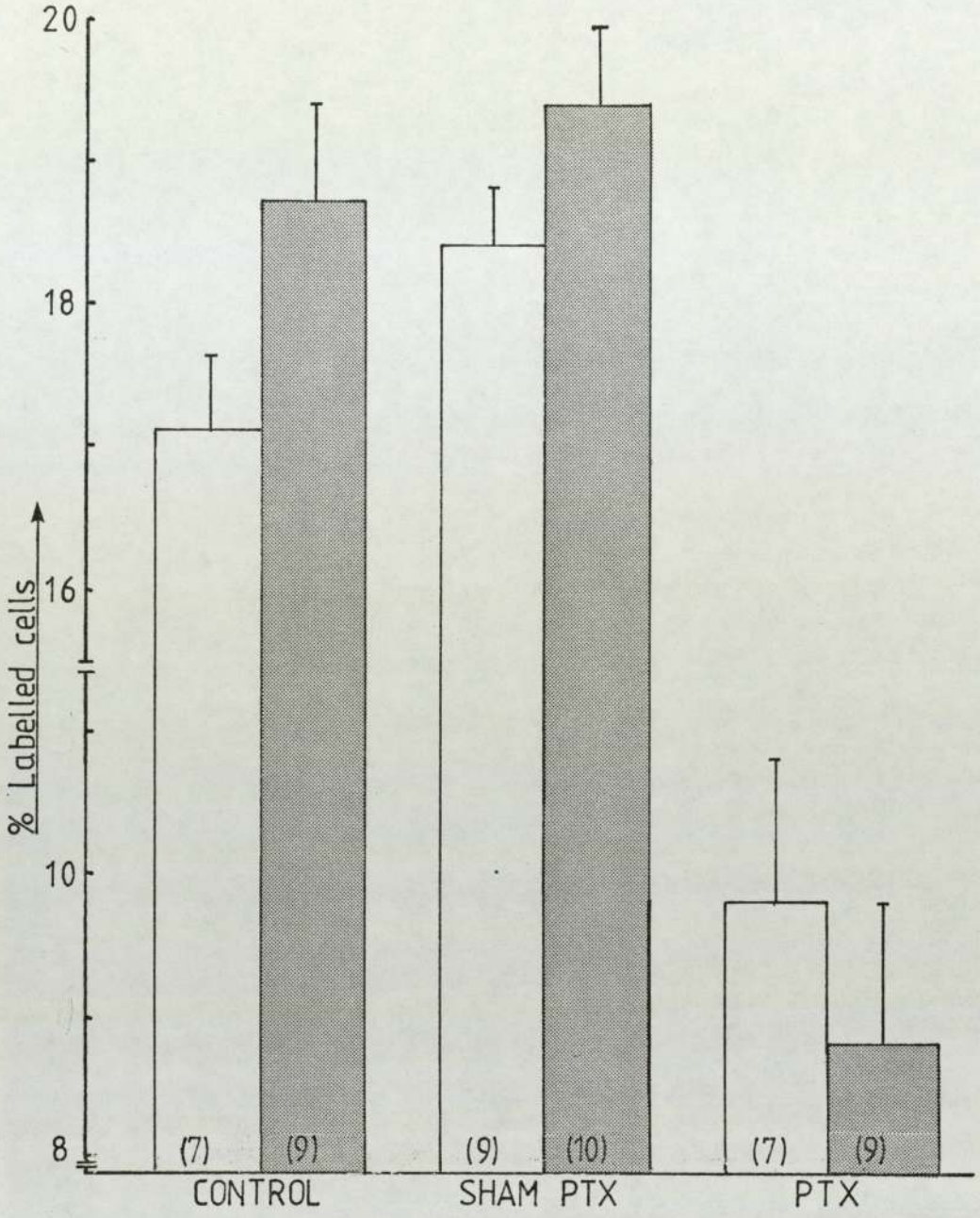


Figure 2.16. The effect of parathyroidectomy (PTX) on the number of bone marrow cells incorporating tritiated thymidine (^{3}HTdR) before (open bars) and after 1ml 10% SRBC in saline (shaded bars). A significant increase in the number of labelled cells was observed in both normal and sham PTX groups ($p<0.05$) while aparathyroid animals failed to mount any proliferative response. The number of cells incorporating ^{3}HTdR in unchallenged animals was significantly depressed by PTX ($p<0.001$). Values are means $\pm$ 1 S.E.M. of between 7 and 10 animals as indicated in parentheses.

control groups, the PTX rats failed to produce any proliferative response. Indeed, the mean incorporation of thymidine by the immunized PTX rats was lower than the unchallenged group. Another point which emerges from these results is that the number of cells which incorporated the DNA precursor in unchallenged PTX rats was much lower than either sham or intact controls ($p<0.001$). This is consistent with the observation that the bone marrow of aparathyroid rats becomes hypoplastic (Rixon,1968).

The spleen has about one tenth the proliferative activity of the bone marrow when measured by either total ^{3}HTdR uptake or autoradiographically. Like the bone marrow parathyroidectomy appears to reduce the number of cells incorporating ^{3}HTdR in the unimmunized group, suggesting that PTX may cause some hypoplasia. Some preliminary data (not shown) supported this notion since PTX appeared to lower the weight of the spleen. The results were inconsistent however, because of inherent variability in spleen size even between rats of the same weight. Interestingly the incorporation of ^{3}HTdR by spleens from sham PTX rats was also reduced when compared to control (fig.2.17). This may indicate a deleterious effect of sham parathyroidectomy.

Following immunization, the number of cells incorporating ^{3}HTdR was significantly increased in control ($p<0.02$), sham PTX ($p<0.05$) and PTX rats ($p<0.05$). This observation is in contrast to that obtained in the bone marrow and indicates that antigen--sensitive splenic lymphocytes were still able to proliferate in the hypocalcaemic environment created by PTX.

The results obtained from autoradiography on bone marrow cells in many respects parallel those obtained by mitotic index. Autoradiography measures the numbers of cells actively incorporating labelled precursor molecules into DNA as this is synthesized.

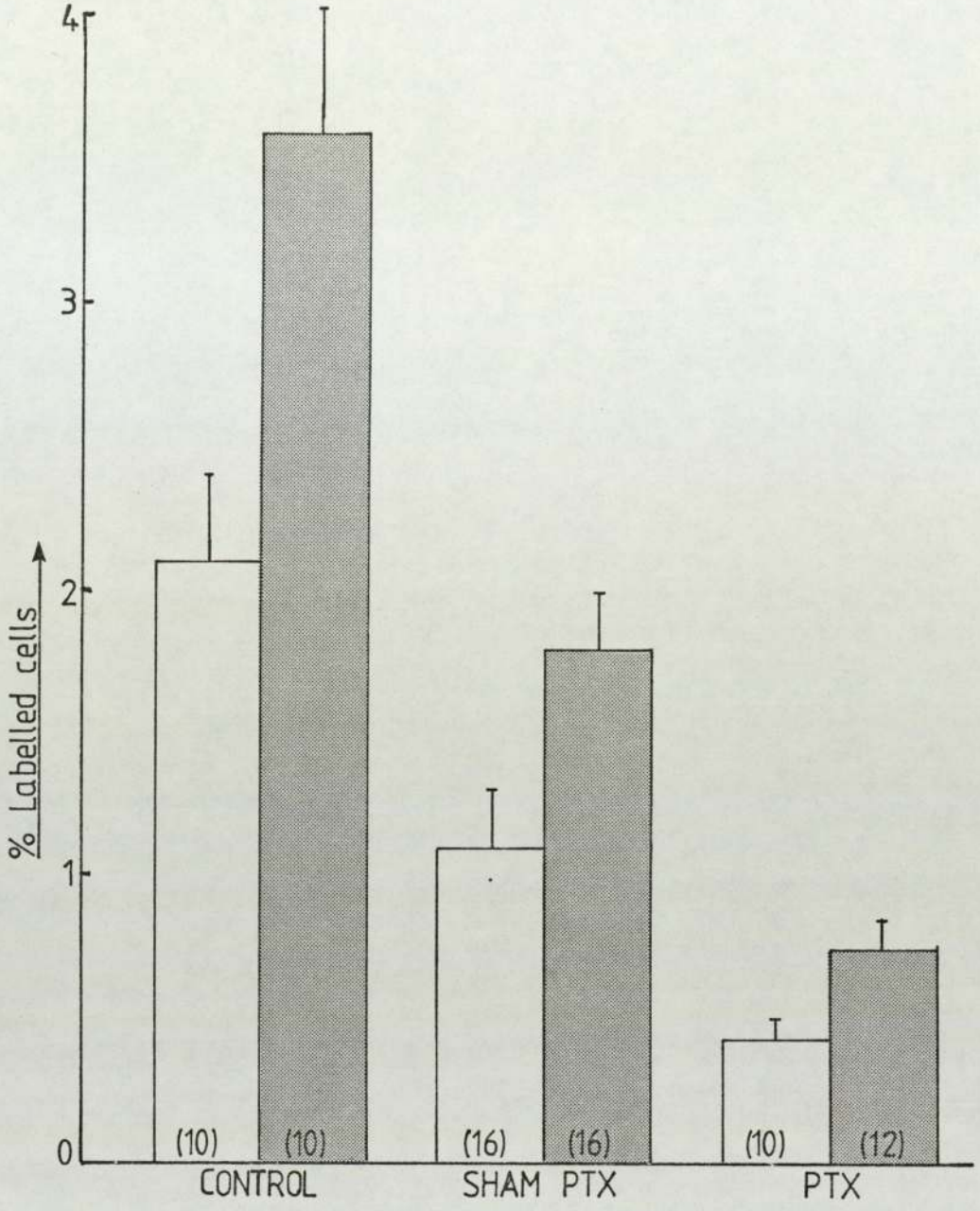


Figure 2.17. The effect of parathyroidectomy on the number of spleen cells incorporating tritiated thymidine (^{3}HTdR) before (open bars) and after 1ml of 10% SRBC in saline (shaded bars). A significant increase in the percentage of labelled cells was observed in intact ($p<0.02$) sham PTX ($p<0.05$) and PTX animals ($p<0.05$). The incorporation of ^{3}HTdR into cells from sham PTX rats was lower than that incorporated by cells from normal rats. A further reduction was evident following PTX. Values are means $\pm$ 1 S.E.M. of 10-16 rats in each case as indicated in parentheses.

An increase in labelled cells following immunization indicates that a larger proportion of cells were in the S phase during the 3 hour incubation period with isotope. On the other hand the MI is a measure of those cells in M at any one time. Given that cells in S proceed through the cycle to complete mitosis, a direct relationship between measurements of cells in S and M would be expected. The advantage of autoradiography is that it measures cells through a longer period of their cycle (and therefore more cells in a cycling population); a feature which is desirable for tissues with a relatively low mitotic activity such as the spleen.

The observation that PTX blocks the antigen-induced proliferative response in the bone marrow but not in the spleen is perhaps indicative of the fundamental differences between lymphoid populations in these two tissues. Although the spleen showed an incremental increase in proliferative activity after challenge, the absolute numbers of cells undergoing DNA synthesis was markedly reduced by PTX when compared with either sham PTX or intact controls (fig.2.17). The experiments and results presented in the next section were initiated to assess the immunological significance of parathyroidectomy.

2.3. The Immunocompetence of Aparathyroid Rats.

The early proliferative phase of the immune response marks the clonal expansion of immunocompetent cells bearing specific immunoglobulin for an antigenic epitope. Abrogation of this phase might be expected to result in reduced effector cell production due to a diminished clonal expansion. This may manifest itself in one or more of several ways depending on the type of antigen challenge. For example, reduced clonal expansion of a pool of antigen-sensitive B-lymphocytes would probably lead to less antibody production while a prolongation of allograft graft survival might result from the formation of fewer cytotoxic T-cells. The observation that aparathyroid rats were unable to mount a normal proliferative response to antigen thus suggested possible immunological insufficiency. It therefore seemed germane to measure the immunocompetence of these animals by choosing assays which tested different facets of the immune response.

The humoral axis of the immune response was measured in two ways. Firstly the concentration of specific anti-SRBC in the serum was measured and secondly the number of cells producing this antibody was assessed from a suspension of splenic lymphocytes using a plaque assay.

In the absence of complement, anti-SRBC causes agglutination of SRBC due to cross linkage of surface epitopes by the antibody. The ability of the antibody to cause agglutination is dependent upon its concentration. Thus a measure of the serum concentration of anti-SRBC is obtainable from mixing SRBC with serial dilutions of serum, and noting when agglutination ceases to occur. This is the principle of the passive haemagglutination assay. In the presence

of complement the binding of antibody to antigen would lead to complement fixation and lysis of the target cells. Serum complement is thus destroyed by heat prior to performing the assay. Since agglutination demands cross-linkage, IgM is most effective because it is pentameric (section 1.6). It follows that the assay is most sensitive to IgM concentration. The addition of mercaptoethanol to the serum dilutions causes the disintegration of the disulphide bonds of the J-chain of IgM thus disrupting the whole immunoglobulin molecule. The agglutination which persists in the presence of mercaptoethanol is thus the result of specific anti-SRBC IgG molecules in the antiserum. The mercaptoethanol-insensitive fraction is thus a measure of the IgG content of the serum.

Blood was obtained by cardiac puncture as described previously (section 2.1) and a portion allowed to clot for 1 hour at room temperature. The serum was normally used fresh though storage at -20°C did not affect the result. The complement of the rat serum was destroyed by heating to 56°C in a waterbath for 30 minutes (Bordet,1920,1939) in a sealed tube. After cooling the samples were recentrifuged so that any small droplets on the inside of the tube which form during heating were re-mixed with the serum sample. A microtitre plate (Cooke) with 'V' shaped wells was prepared by placing 25 µl of saline in each hole from a dropping pipette (Cooke). A 25 µl sample of serum was taken from the tube with a diluting tulip (Cooke) which had been previously purged and calibrated. A serial dilution of the serum sample was prepared discarding the sample from the last hole. In such a series the serum concentration at hole 1 is 2^{-1} (= 1/2 x serum concentration), at hole 2 it is 2^{-2} (1/4) and 3 is 2^{-3} (1/8) etc. Thus the hole number corresponds to $-\log_2$ serum concentration, and was used directly to plot on the graphs. Four rows of dilutions were made for each serum sample.

To two rows was added one drop (25 µl) per well of 0.9% saline while in the other two rows an equal volume of 1M mercaptoethanol was added. Finally 25 µl of a 1% suspension of saline-washed target cells (SRBC) was added to all wells. The tray was shaken gently, sealed with clear film and incubated for 1 hour at 37°C then overnight at 4°C. The titre of the antibody was taken as the last hole in which agglutination had taken place. Agglutination appears as a halo around the centre of the well while non--agglutinated erythrocytes form a tight "button" in the centre of the well. The diluting tulips were washed in 2x distilled water, purged in a bunsen and re-checked for accurate dispensing of 25 µl between each serum sample using proprietary calibration cards (Cooke).

The results are shown in figures 2.18, 2.19 and 2.20. Following immunization there is a lag period of two days but on the third day the total agglutinin titre is begining to rise (fig.2.18). At this point the agglutination is caused almost exclusively by antibody of the IgM class. Thus at day 3 the mercaptoethanol (ME) sensitive fraction of the antibody (i.e. IgM) is present in higher concentration (fig.2.19) than the ME-insensitive (IgG) fraction (fig.2.20). The results show that both PTX and sham PTX initiate the response to SRBC at the same time and that antibodies are detectable in the sera at the same time. The peak titre for total agglutinins (fig.2.18) is reached at about day 6 and the fall in titre is slow. Thus an appreciable titre remains 2 weeks after the initial challenge and indeed total agglutinin titre of 1-3 was consistently observed up to three months after a single challenge of SRBC (data not shown). At no point in the primary immune response to SRBC was the response of aparathyroid animals significantly less than the sham operated groups. However, it should be noted that

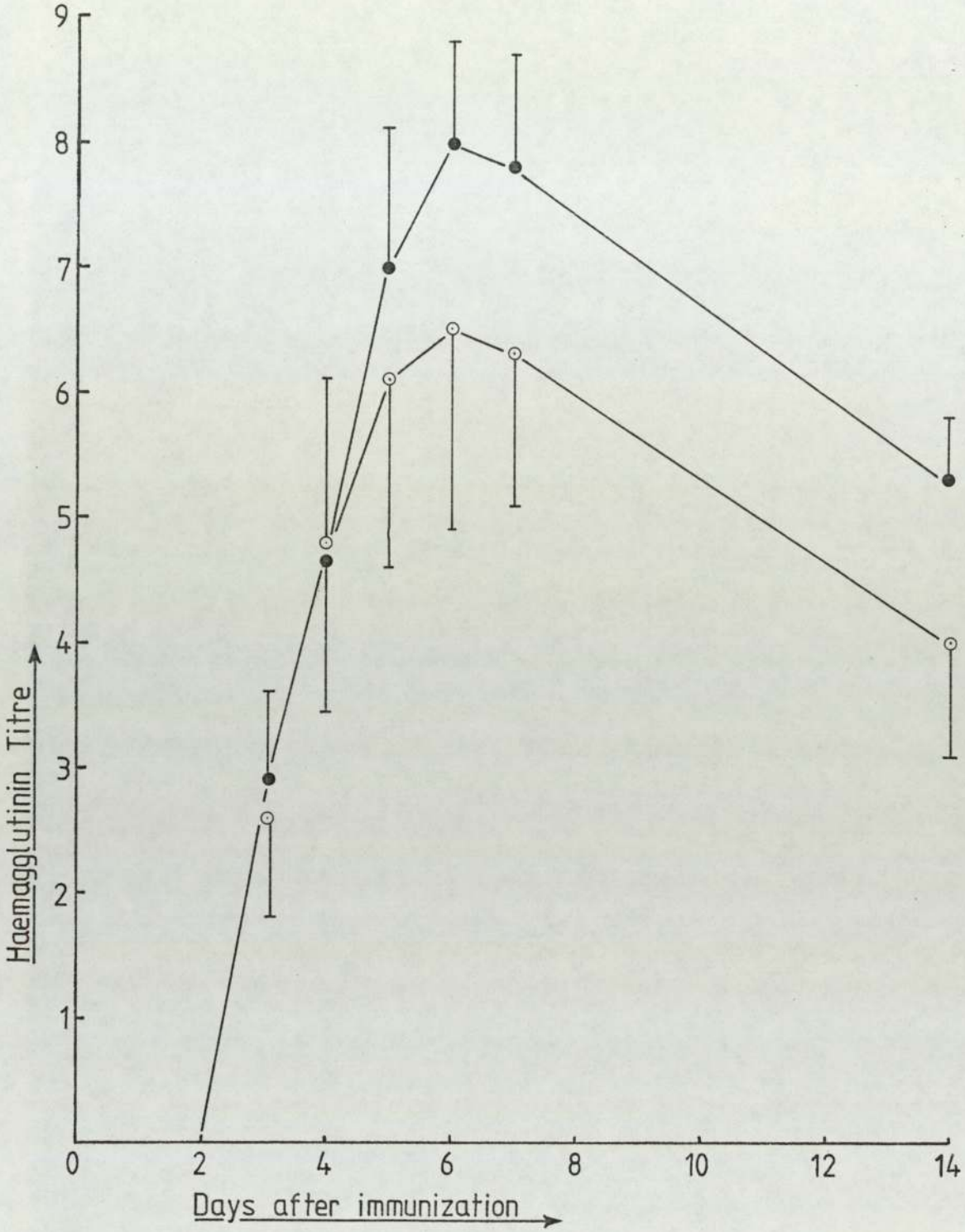


Figure 2.18. Total agglutinating antibody titre assessed by passive haemagglutination following a single i.p. injection of SRBC given on day 0. The response of PTX (o-o) was compared with that of sham PTX (●-●) over the first two weeks of the primary response. Although the mean titre for PTX is lower than sham, both at the peak response and thereafter, there is no significant difference between the points on any day. Values are mean ± 1 S.E.M. of duplicate samples from eight animals in each case.

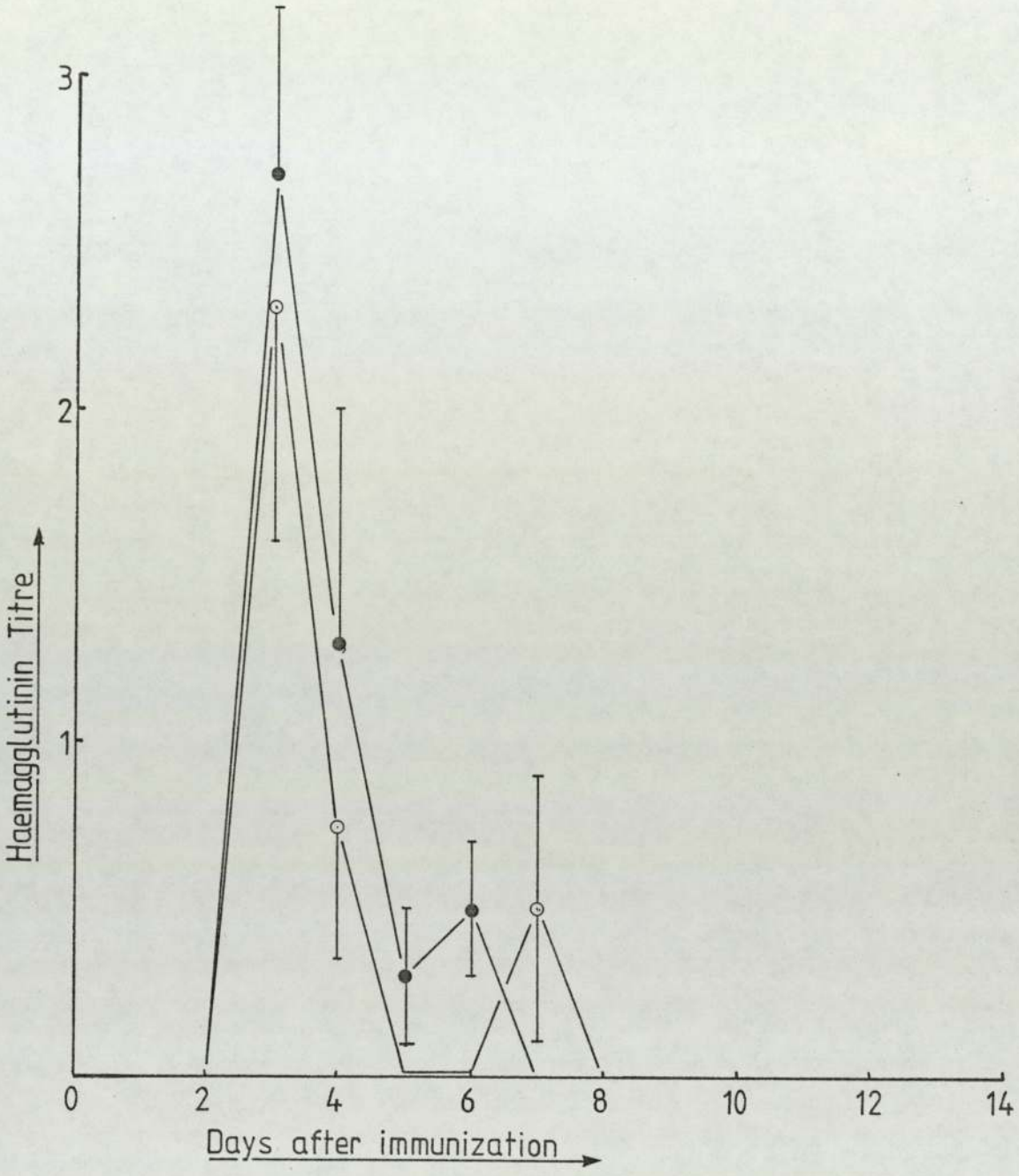


Figure 2.19. The specific IgM response (ME-sensitive antibody) of sham PTX (●-●) and PTX (o-o) rats to a single injection of SRBC on day 0. A rapid increase in titre was observed between days 2 and 3 but this was transitory and only low levels persisted from 5 days onwards. There was no significant difference between sham PTX and PTX at any point during the primary response. Values are means ± 1 S.E.M. of duplicate samples from eight animals.

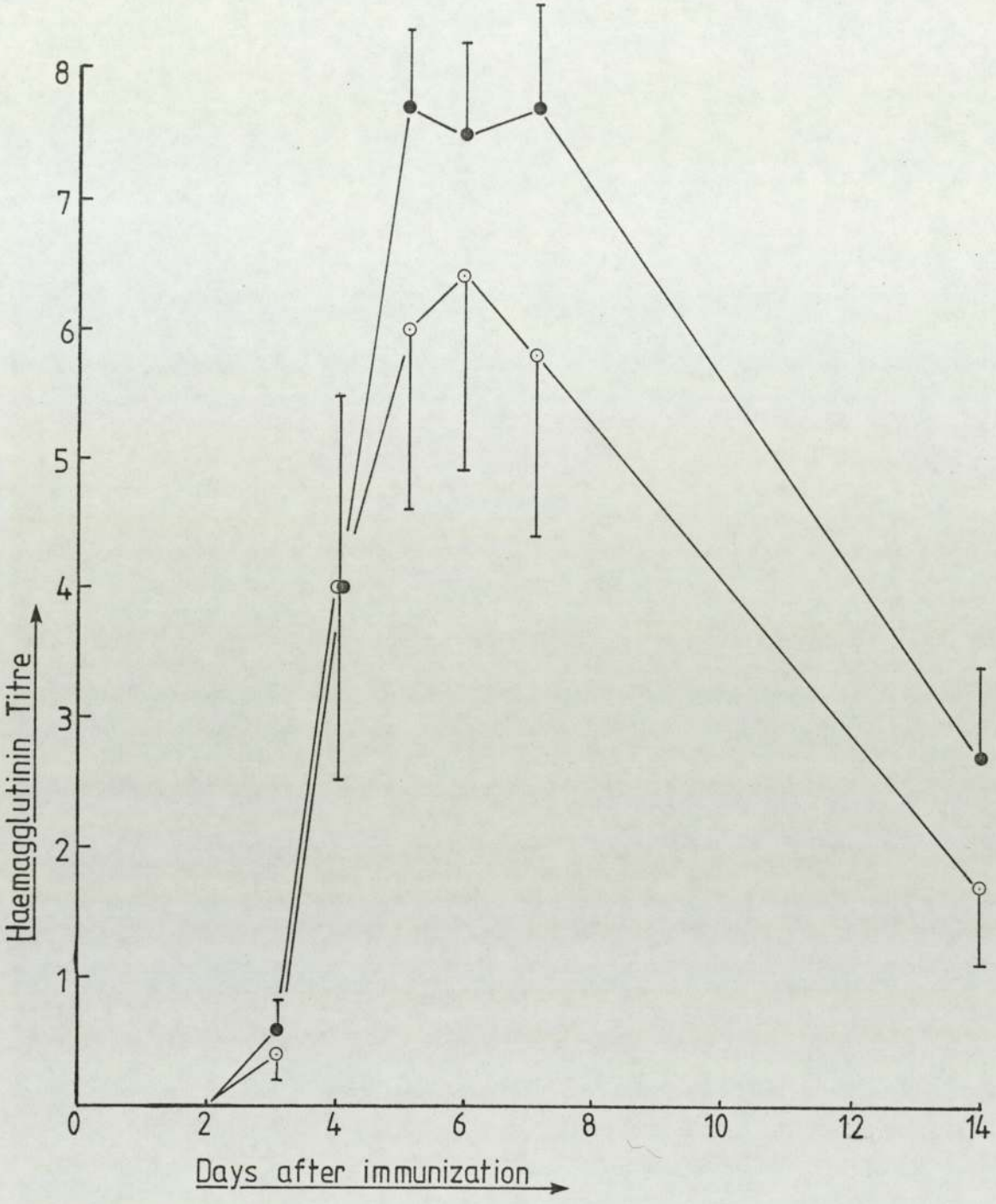


Figure 2.20. The specific IgG response (ME resistant antibody) of sham PTX (●-●) and PTX (o-o) to a single injection of SRBC on day 0. In both groups the response starts at the same time and rises at the same rate. The maximum mean titre achieved by the PTX group is lower than the sham PTX group and remains thus for the rest of the primary response though there is no statistically significant difference between any point. Values are means ± 1 S.E.M. of duplicate samples from eight animals in each case.

the mean peak response of PTX rats is less than the sham operated and the antibody titre remain lower for the remainder of the primary response.

As mentioned previously the IgM response occurs very early (fig.2.19), exhibits a sharp peak on day 3 and declines almost as rapidly. The IgM responses of sham PTX and PTX are totally indistinguishable from one another.

The titre of serum IgG in the primary response to SRBC (fig.2.20) follows closely that of total haemagglutinating immunoglobulins. There is no significant difference between the responses of sham PTX and PTX rats. Even so, the mean titre for IgG is consistantly one hole lower in the PTX groups from day 5, as the titre reaches maximum, onwards. Taken simply this would represent twice as much specific antibody in the sham PTX group. In fact the haemagglutination assay is not sufficiently sensitive to adequately distinguish a difference of only one hole. However, the antibody titre of aparathyroid rats was found to be consistently lower than sham PTX. Taken over the whole response to a given number of SRBC's, this probably indicates impairment of antibody function in parathyroidectomized animals. Whether this would affect the ability of PTX rats to resist infection cannot be ascertained from this result alone.

While the foregoing test measures the concentration of a specific serum antibody and is thus an important immunological assay, it is not possible to know how many plasma cells are responsible for the production of the immunoglobulin. In order to ascertain this parameter an adaption of the Jerne plaque technique for innumerating antibody-forming cells was used (Jerne, Nordin & Henry,1963). Unlike the original assay in which cells were suspended in moulten agar this modification makes use of cells

suspended in ordinary buffered medium in a monolayer of target cells (Cunningham,1965; Cunningham & Snzezbern,1968). The regimen is summarized in figure 2.21. The spleen was removed from an immunized animal and minced in cold medium 199 buffered with HEPES and supplemented with 10% foetal calf serum. The cell suspension was washed and resuspended in cold 0.83% ammonium chloride (tris buffered) at pH 7.2 which provides a hypotonic shock to the erythrocytes (which thus lyse) without toxicity to the lymphocytes (Boyle,1968). After 10 minutes the cell suspension is centrifuged, washed twice in fresh medium and resuspended in an appropriate volume. A cell count was performed on a electronic cell counter (Coulter electronics) with Zaponin to remove the few contaminating erythrocytes which remain (details in section 2.2). Viability of the lymphocyte suspension was assessed by ability to exclude the dye trypan blue. One drop of 0.4% solution of trypan blue in balanced salt solution (Flow) was added to 0.5ml. aliquot of the cell suspension. Viable cells exclude the dye while dead cells are stained blue. The number of unstained cells expressed as a percentage of the total population (i.e. % viability) was estimated from samples placed in an improved Neubauer counting chamber between 5-15 minutes after addition of the dye. Two 0.5ml. aliquots of the cell suspension were pipetted into two small tubes. Then 0.1ml. of 30% suspension of saline-washed SRBC (target cells), 50 µl of fresh guinea-pig serum (stored at -20°C) as a source of complement was placed in each tube. This was followed by either 25 µl of rabbit anti-rat IgG (Miles) or 25 µl of medium (to keep the final volumes the same). The mixture was then placed in special chambers called Cunningham chambers. These were manufactured by sticking two acid alcohol cleaned microscope slides together with double-sided adhesive tape (4000D, J.Kilby & Son). The mixture was

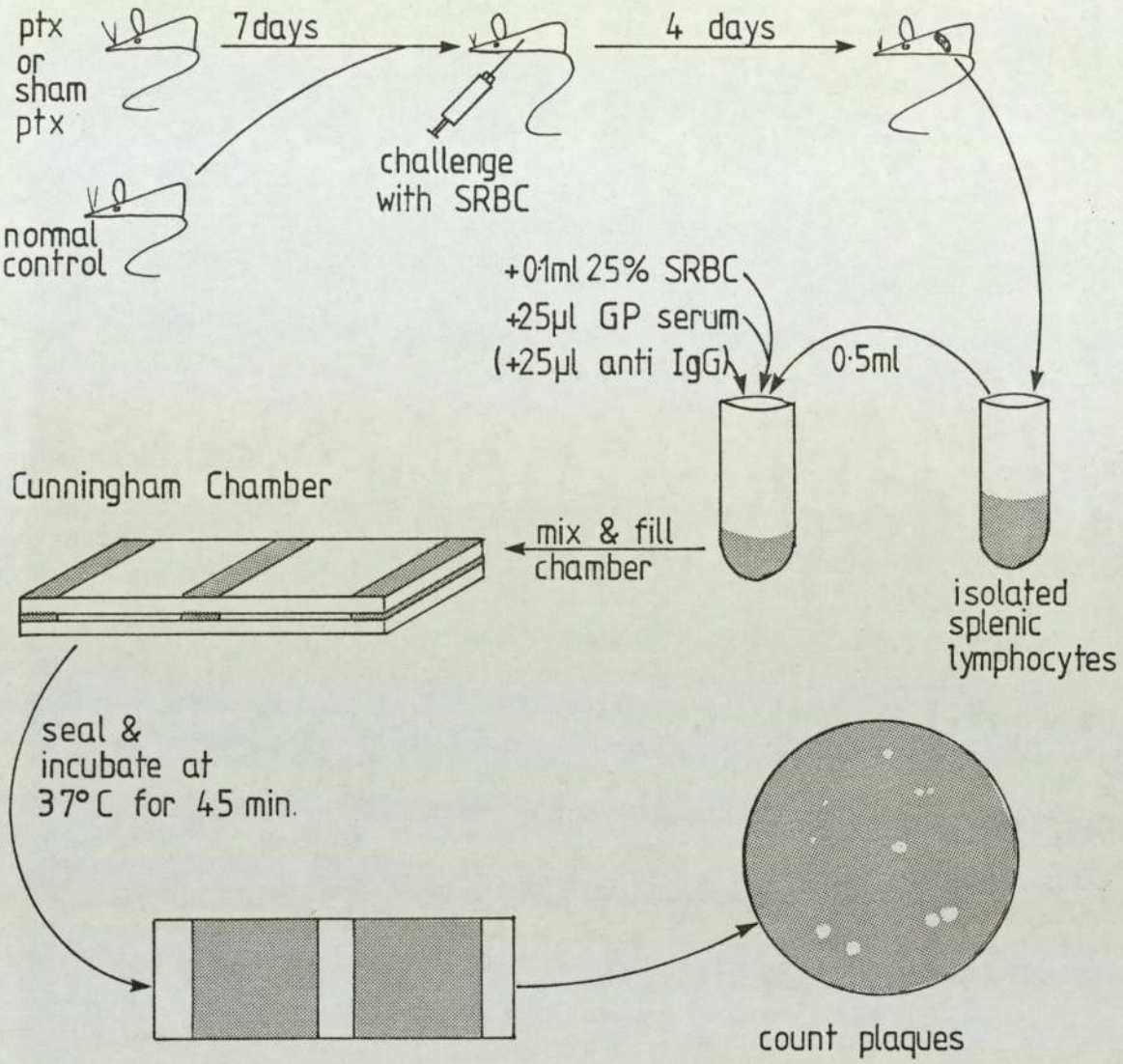


Figure 2.21. The Cunningham plaque assay for antibody secreting cells. The assay involves suspending splenic lymphocytes from rats previously immunized with sheep erythrocytes (SRBC) in a monolayer of SRBC in the presence of complement. Fresh frozen guinea pig (GP) serum was used as a source of complement. Discrete areas of lysis develop around those cells secreting specific anti SRBC antibody since this will bind to the target cell and fix complement causing lysis of the erythrocyte. Normally the technique only detects IgM-secreting cells (direct plaques) since this fixes complement most efficiently (see section 1.6). However, the inclusion of Goat anti-rat IgG, at a suitable concentration, allows the detection of IgG-secreting cells also (indirect plaques) by binding together two IgG molecules thus creating one complement fixation site (see section 1.6). The calculations required for the plaque assay and titration of the antiserum are described in appendix 2.

poured into a glass trough from which the chambers were filled by placing the edge of the slides into the liquid which then flows into the chamber by capillarity. Duplicate chambers were filled from each tube giving four countable areas per sample (fig.2.21). The chambers were incubated for 45 minutes at 37°C after sealing the chambers to prevent evaporation by dipping the extreme edges into a paraffin wax/petroleum jelly mixture (1:1) at 70°C.

During the incubation, specific rat anti-SRBC is secreted from some plasma cells in the chamber and this diffuses out into the liquid between the slides. The antibody binds to the SRBC target cells, fixes complement and causes lysis of the SRBC. Thus discrete areas of lysis or plaques, develop in the monolayer of SRBC around those plasma cells producing the appropriate antibody. From a knowledge of the dimensions of the Cunningham chamber and the number of live cells placed in it, the number of antibody producing cells per million cells can be calculated (appendix 2.) IgM, is a powerful haemolysin in the presence of complement (section 1.6). In contrast anti-SRBC of the IgG class would be unlikely to cause lysis unless present in high concentrations because of the requirement for two complement binding sites in close proximity to result in complement fixation. The addition of anti-rat IgG to the cell suspension results in the binding together of two rat IgG molecules secreted by the plasma cells. Those IgG molecules thus joined which are specific for an SRBC epitope will bind to it so that complement fixation and lysis can then proceed. Thus the slides containing anti rat IgG will have plaques which are caused by IgG as well as IgM. The IgM plaques are called 'direct' plaques while the IgG plaques are 'indirect' because a developing antiserum has to be added. Unfortunately a simple subtraction of the numbers of plaques obtained without antiserum from the numbers obtained with

does not give the number of indirect plaques because the antiserum used invariably inhibits direct plaque formation. Two titrations of the developing antiserum were therefore performed in order to find a suitable working dilution for the antiserum. The method and results are detailed in appendix 2. The dilution chosen was 1/2040 (final dilution) which was equivalent to a final concentration of 2.88 µg/ml.

Since the cells are supported in liquid medium, they are subjected to Brownian motion which causes the plaques to fade after a few hours. Though this problem can be overcome by counting the slides within strict time limits, it was found convenient to photograph the slides so that counting could be done at leisure. Recounting of slides was also made possible by adopting this method. A half plate enlarger was adapted to hold four Cunningham chambers side by side in the negative carrier. The erythrocyte monolayers thus took the place of the negative of a film and by direct printing onto grade 4 high-contrast paper (Kodak) the plaques were visualized as dark spots. The occasional plaque-sized bubble did not interfere with the count because due to their refractive properties bubbles were surrounded by a white ring in the printed photograph. A magnification of 3x was achieved and a perspex sheet etched with 1cm squares placed on the paper during printing provided a grid on the photograph which further aided counting within a strict area. Using this technique the number of direct and indirect plaque forming cells were measured in normal, sham PTX and PTX rats following 1ml/200g of a 10% suspension of saline-washed SRBC.

In a preliminary experiment it was found that the numbers of direct plaque forming cells in the spleen reached a maximum on day 4. The distribution of these cells in the lymphoid tissues on day 4 is shown in figure 2.22. Splenic lymphocytes and bone marrow cells

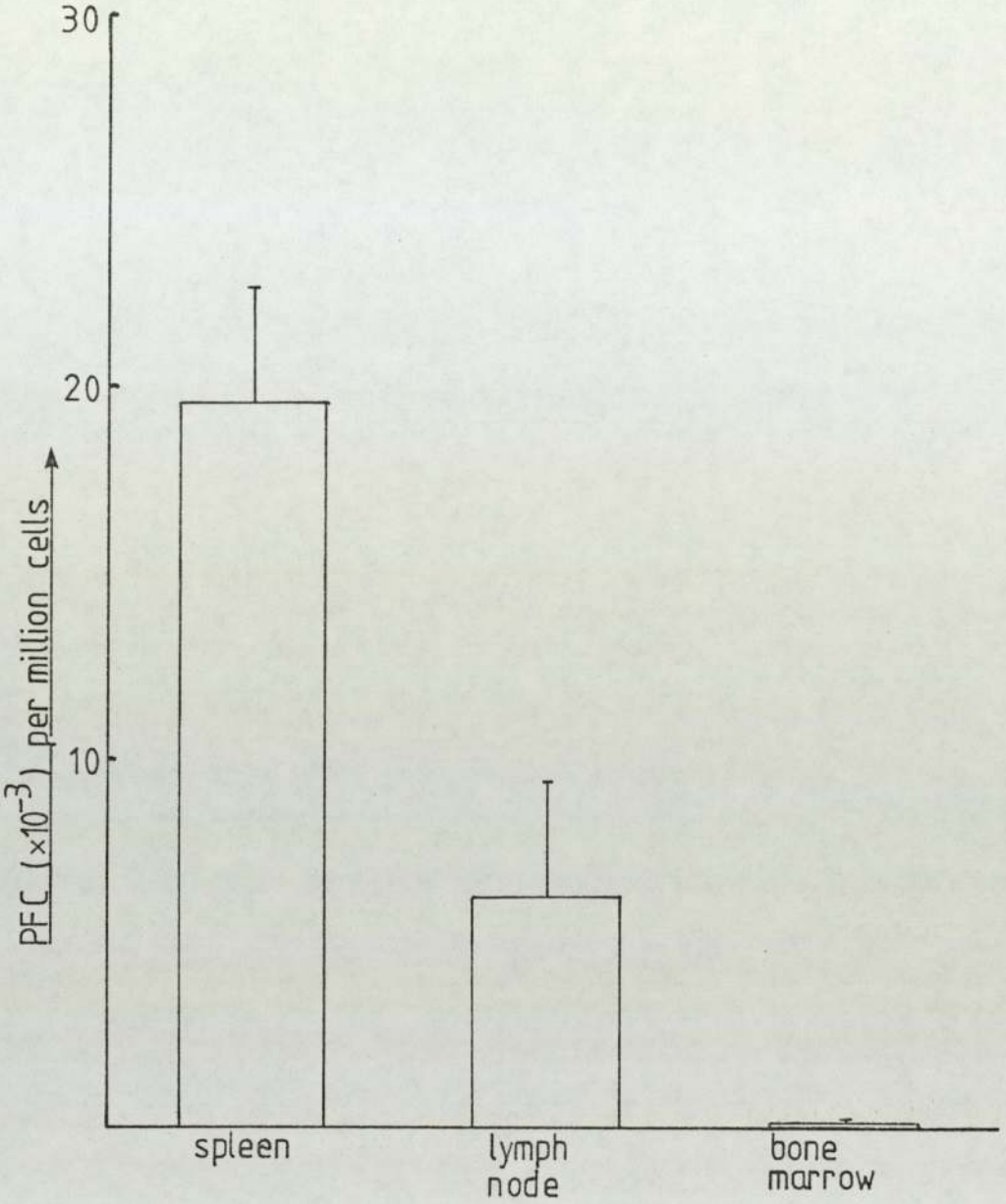


Figure 2.22. The distribution of plaque forming cells in normal rats on the fourth day of a primary response to SRBC. Lymph node cells were obtained from pooled mesenteric lymph nodes. It is evident that the spleen is likely to be the major source of antibody at this time while the bone marrow contains negligable numbers of PFC. The values represent means $\pm$ 1 S.E.M. of six animals in each case.

were obtained as previously described. Mesenteric lymph node cells were obtained by teasing a pool of nodes in cold medium and removing the unicellular suspension of lymphocytes after the debris had settled for 2 minutes. It can be seen that the spleen represents the largest source of plaque forming cells andhence antibody with the lymph nodes also producing appreciable amounts. However, there were very few antibody-forming cells detectable in the bone marrow. The spleen was therefore chosen as the routine source of lymphocytes for the assay of PFC in intact, sham PTX and PTX rats.

The results obtained are summarized in figures 2.23 and 2.24. After a lag period of about two days there was an explosive rise in the number of plaque forming cells indicating an almost synchronous arrival of lymphocytes into the antibody secreting phase. The number of direct plaques (fig.2.23) reached a peak on day 3 which lasted until day 4 and declined thereafter to very low levels by day 11. The response of both sham PTX and PTX rats was essentially similar to the normal animal but there was a tendency for later onset and lower peak. Thus in both sham PTX and PTX groups the peak was obtained on day 4 only. The response of PTX rats was at no point significantly less than sham PTX but both groups had a significantly lower response compared to intact animals on day 3.

The results obtained for indirect (IgG) secreting cells during the primary response (fig.2.24) were similar to the result for direct plaques. Thus in intact animals a rapid increase in numbers of indirect plaques occurred and following a peak on day 4 the numbers fell to low levels by day 7. The pattern of development of indirect plaques in sham PTX and PTX rats paralleled the response of intact animals. Again there was a tendency for the appearance of direct plaques to be delayed by one day in both PTX and sham PTX groups. There were significantly fewer plaques in PTX and sham

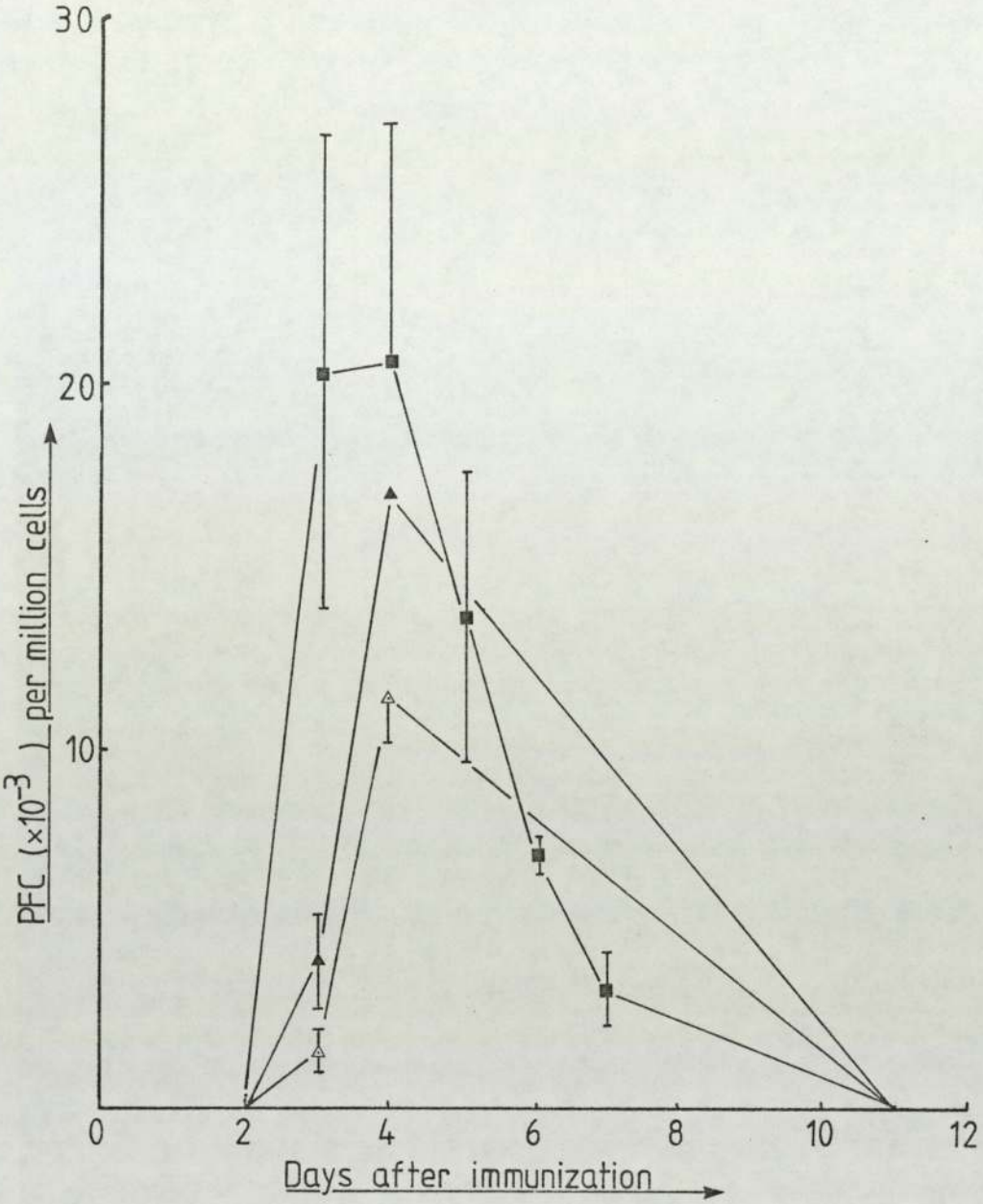


Figure 2.23. The development of direct plaques (IgM) in the spleen of normal (■) sham PTX (▲) and PTX (△) rats following immunization on day 0 with SRBC. There was a tendency for PTX rats to show a slightly reduced response with later onset and lower peak than normal animals. However, the response of PTX rats was not significantly lower than the sham control group. Values are means ± 1 S.E.M. of between 4 and 8 animals in each case.

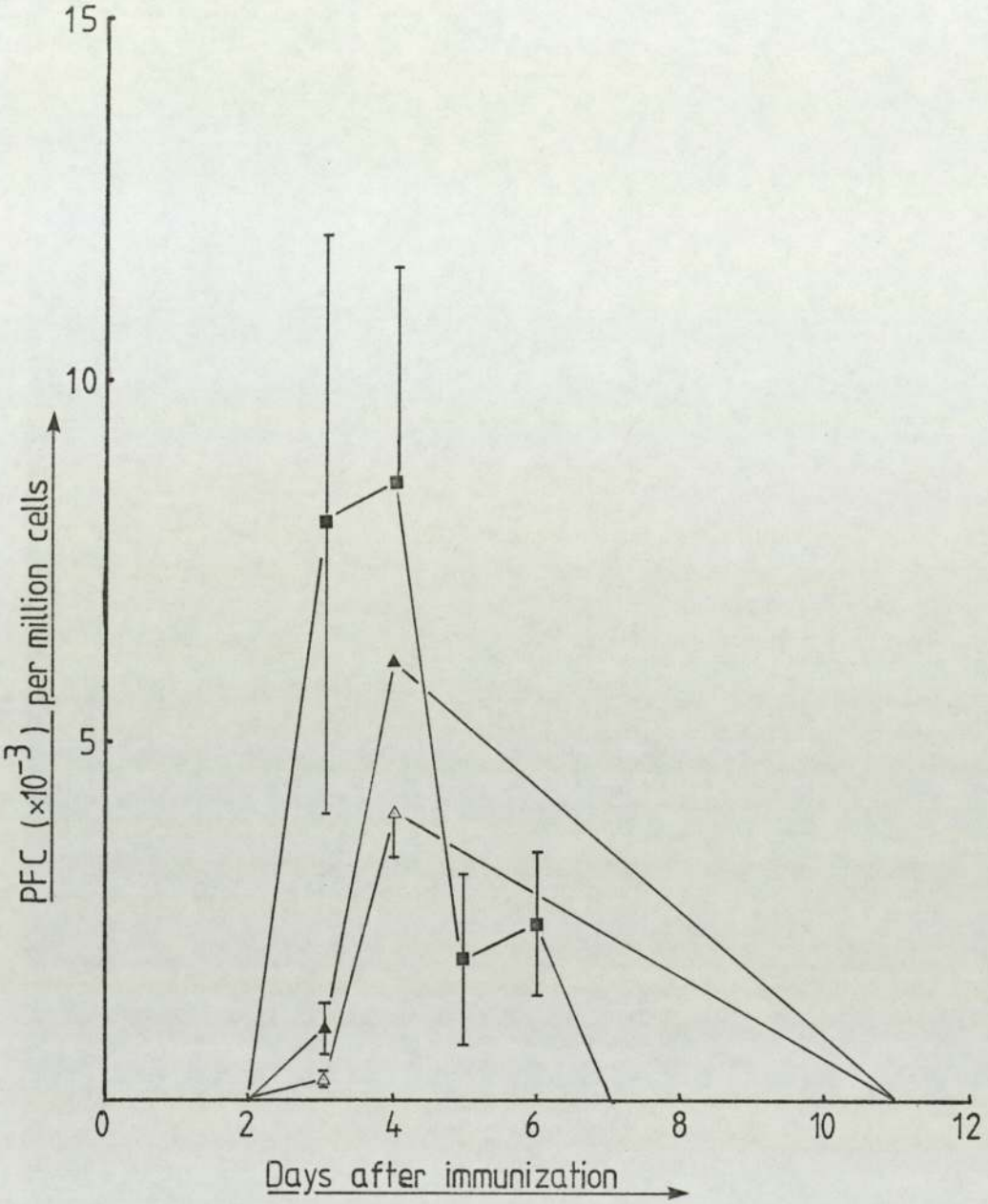


Figure 2.24. The development of indirect plaques in the spleen during the primary response to SRBC in normal (■) sham PTX (▲) and PTX (△) rats. A similar pattern was evident to that obtained for direct plaques. At no point is the response of PTX rats significantly lower than sham PTX even though the mean value is consistently lower throughout the response. The responses of both sham PTX and PTX are suppressed on day 3 with respect to normal controls. Values obtained are means ± 1 S.E.M. of between 4 and 8 animals in each case.

PTX animals on day 3 compared with intact animals but there was no significant difference between the responses of sham PTX and PTX at any point.

The thymus exhibits severe steroid-independent involution following PTX (Perris, et al 1970) and this organ has been implicated either directly or indirectly in cell mediated immune responses (CMI) (see section 1.6). It was possible that parathyroidectomy may act as a functional thymectomy and in addition affect T-cell function in secondary lymphoid tissues. Alternatively it was possible that an impairment of CMI might be the result of a defect of sub-classes of T-cells rather than on T-cells in general. The CMI of aparathyroid rats was thus assessed by various means described below and compared with control groups.

The delayed hypersensitivity response (DH) to oxazolone is a typical CMI response which requires T:T cell interaction. Both helper T-cell (T_H) and suppressor T-cell (T_S) involvement has been demonstrated in this response. (Bullock, Katz & Benacerraf,1975; Parker, Turk & Scheper,1976; Katz,1977b; Liew,1977). When applied topically to pre-sensitized animals, this substance causes erythema and oedema after a delay of some hours and the response persists for a few days (fig.2.25). As the response is quantitative over a concentration range it is possible to use the test as measurement of an animal to mount a delayed hypersensitivity response. Although oxazolone will dissolve in warm ethanol, a preliminary trial showed this solvent to be unsatisfactory. If oxazolone was applied to the ear in alcohol the solvent rapidly evaporated and left a crust of oxazolone on the surface of the ear which could easily have been scratched off by the animals. It was therefore decided to use warm arachis oil (BDH) as an alternative solvent as this substance soaked into the skin of the ear and did not leave the oxazolone exposed.

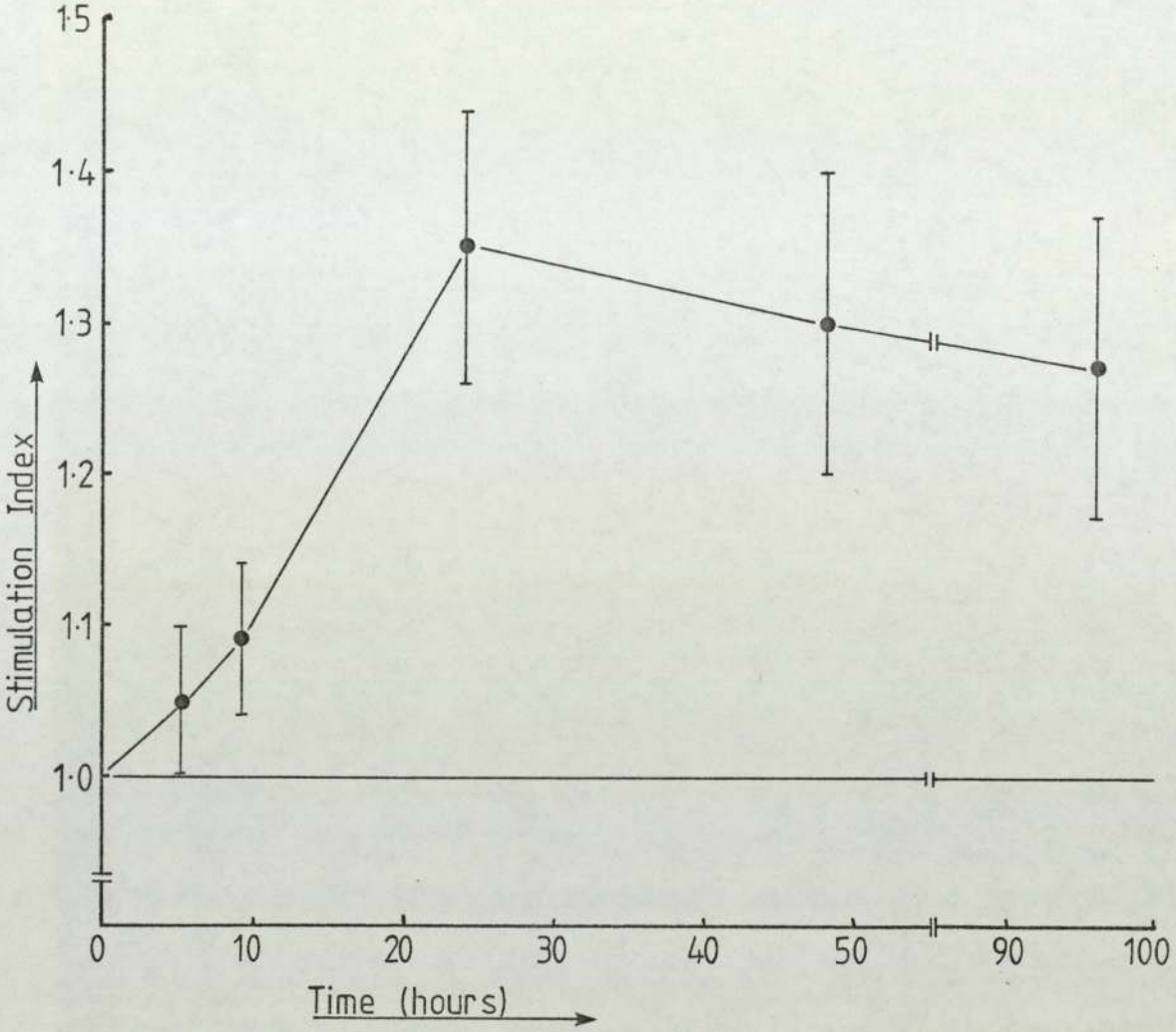


Figure 2.25. The delayed type hypersensitive response to oxazolone in sensitized rats. Oxazolone (2mg in arachis oil) was applied topically to the posterior surface of the ear. Seven days later the same site was rechallenged and the thickness of the ear measured using a micrometer. The stimulation index (test/control) reached maximum at 24 hours and then declined slowly over several days. Values represent means ± 1 S.E.M. of six animals in each case.

The ears of rats were sensitized by applying 2mg oxazolone in 20 µl of arachis oil with a micropipette. After 7 days the animals were challenged again with the same dose at the same site and the thickness of the ear measured during the development of the response to ±0.1mm using an engineers micrometer. It was felt that contra--lateral ear thickness might not prove adequate because of possible self contamination with the stimulant. Consequently control values for ear thickness were obtained from a large number of animals treated with arachis oil only. The Stimulation Index (SI) was calculated from:-

$$SI = \frac{\text{Test Thickness}}{\text{Control Thickness.}}$$

Using this procedure the time taken for the maximal development of the oedemic response was estimated. The results (fig.2.25) show that the swelling develops to maximum in 24 hours and gradually declines over the next few days. The dose/response curve at 24 hours (fig.2.26) showed that the SI bore a linear relationship to the log dose over the range 10µg -10mg. A standard dose of 4mg oxazolone in 40 µl of arachis oil was chosen to test the ability of parathyroidectomized animals to mount a delayed hypersensitivity response.

Following challenge with 4mg oxazolone of normal animals sensitized with 2mg oxazolone 7 days earlier the thickness of the ear increased by about 40% (fig.2.27). The control (unstimulated) ear thickness in both sham parathyroidectomized (Sham PTX) and parathyroidectomized (PTX) animals was slightly higher than normal controls (SI = 1.06 approx.). In both sham PTX and PTX the incremental increase was less than the normal unoperated control animals. In the sham PTX group the response was elevated by 14% over the untreated control. In the PTX group the swelling represented a 24% increase over the untreated control. (fig.2.27).

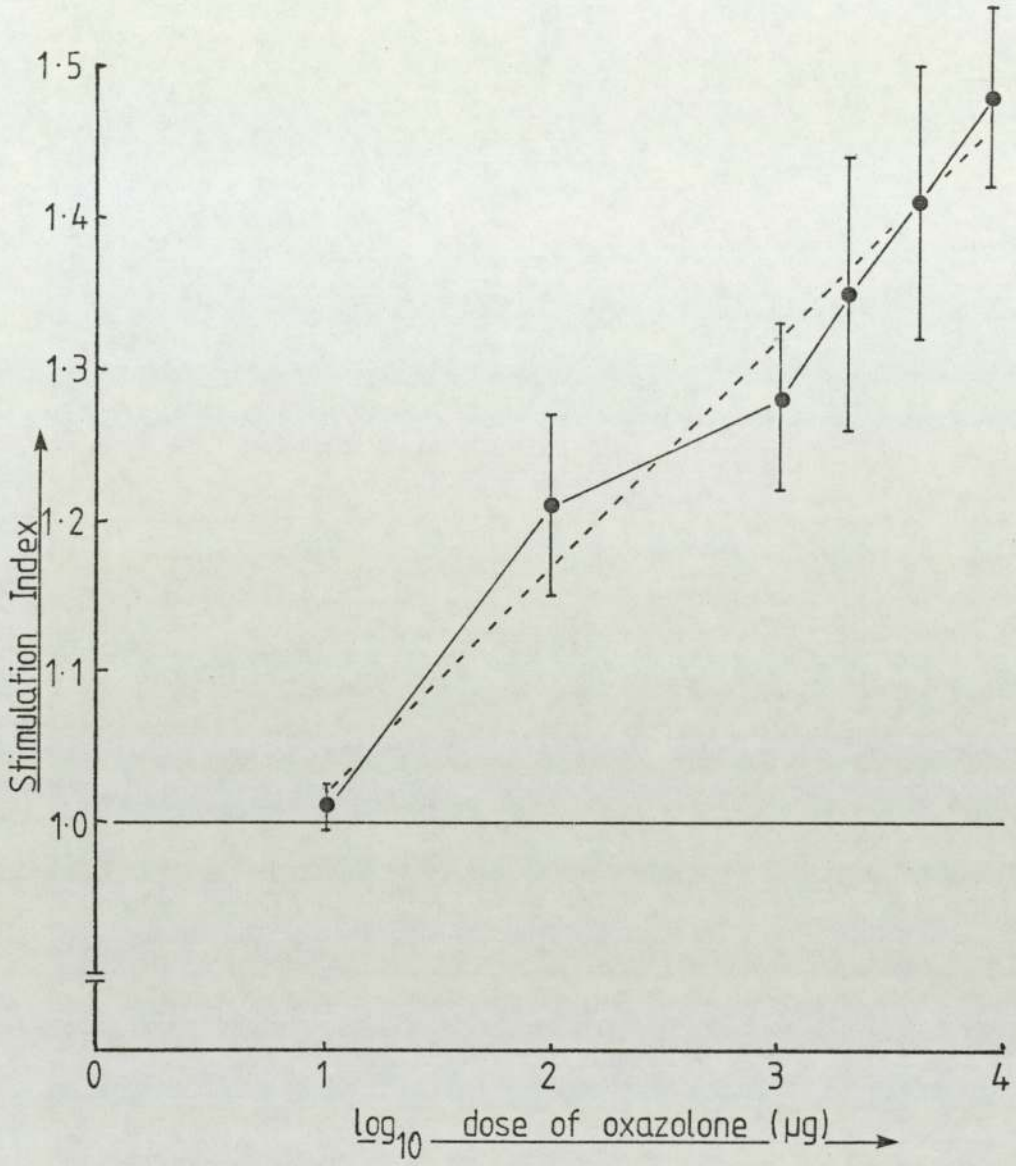


Figure 2.26. The dose response curve for oxazolone. A variety of doses between 1µg and 10mg in 20µl of arachis oil were used to challenge animals sensitized with 2mg oxazolone 7 days earlier. The response, represented as the Stimulation Index, showed a linear relationship to log dose. The broken line represents a line fitted by linear regression (least squares) where r[reg]=0.985. All values are means ± 1 S.E.M. of 4 animals.

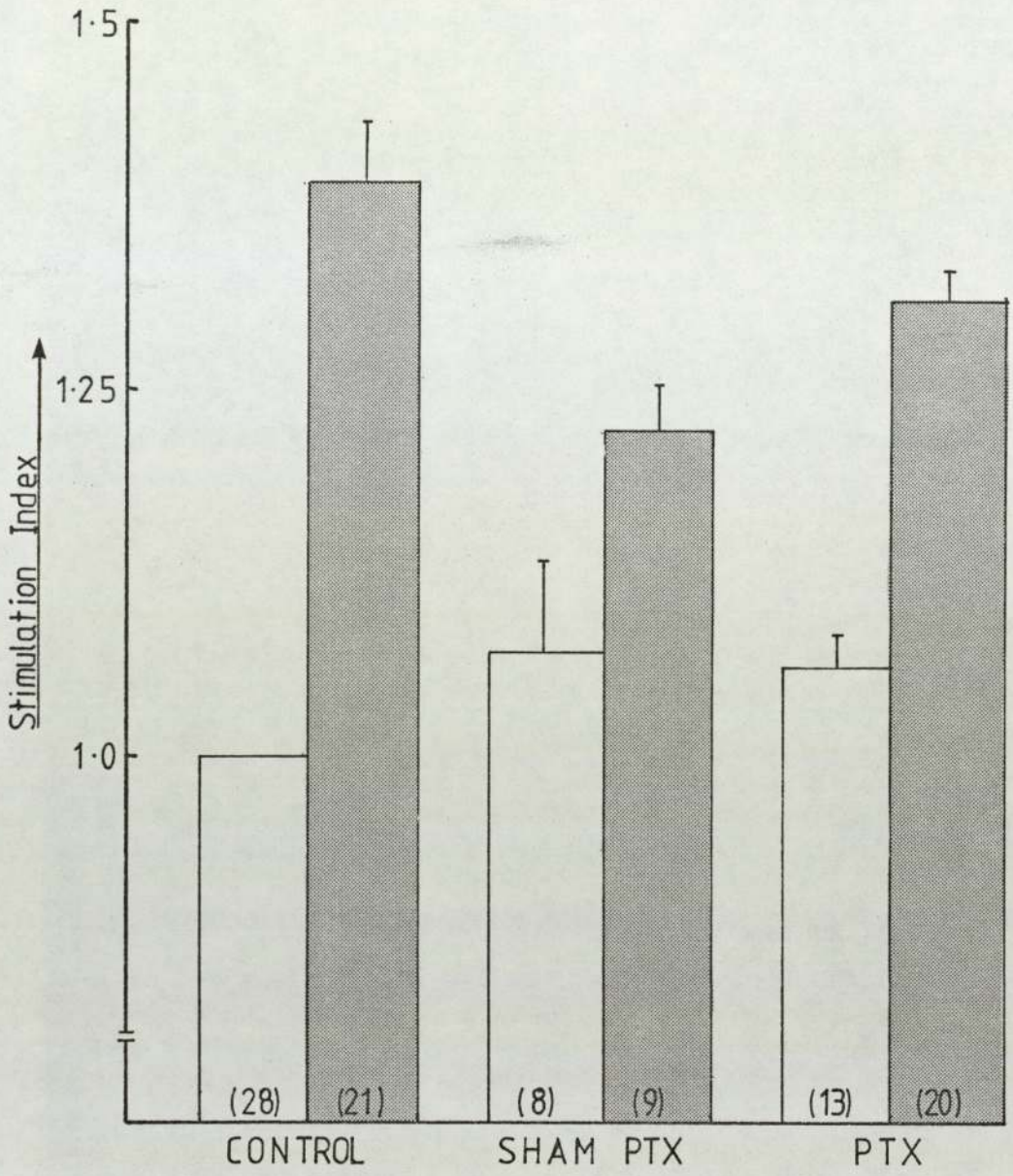


Figure 2.27. The effect of parathyroidectomy on the delayed hypersensitivity response to oxazolone. All groups show significant stimulation ($p<0.001$) in the challenged group (shaded bars) when compared to unchallenged but sensitized controls (open bars). Compared to normal controls, the response of sham PTX is significantly depressed ($p<0.05$) but the response of PTX animals is not depressed ($0.1<p<0.05$). The response of PTX animals is significantly greater than sham PTX ($p<0.05$). The bars represent means $\pm$ 1 S.E.M. and the number measurements in each case are shown in parentheses.

It is not clear why both sham PTX and PTX groups should respond less than the normal controls but perhaps this represents some residual stress from the operation (though the time of sensitization was 7 days post-operative). Since the sham PTX group forms a more accurate control for the PTX results it would appear from the data presented that aparathyroid animals respond to oxazolone by enhanced swelling of the ear ($p<0.05$). This result suggests the possibility of reduced numbers of T_S cells in the aparathyroid group.

In a preliminary experiment using skin xenografts it was found that parathyroidectomy prolonged the survival time from a mean of 9 days to greater than 13 days. The method of performing the xenograft is given in section 2.1. This implied a deficiency in CMI response of the animals. To investigate this further the versatile popliteal lymph node assay (Ford, Burr & Simonsen,1970) was used. The assay is a modification of the spleen weight assay (Simonsen,1962) used for mice. The incremental increase in spleen weight in rats is for some reason not as great in rats as it is in mice and it has been found convenient to use the popliteal lymph node instead (Ford, et al.,1970). This lymph node lies just behind the knee joint and in unchallenged animals weighs a mere 4-6mg (fresh wet-weight). The node can be visualized by an injection of 0.1ml of Evans Blue dye into the foot pad 5-10 minutes before sacrifice which travels up the lymphatics and stains the node dark blue.

If rats of strain A are crossed with strain B then the resulting F_1 offspring, AB, will accept grafts from either parent (P) since the surface antigens A or B on parent donor cells are recognized as self by the AB recipient which has both A and B on its cell's surface. However, A cells (from P_A) are able to recognize AB as foreign. If the donated cells are lymphocytes from parental

animals then these cells will mount a response against the F_1 host, while the host AB, will be unable to reciprocate. The result is a graft-versus host reaction (GVHR). If parental lymphocytes, prepared as described previously, are injected into the foot pad of F_1 strain rats then these lymphocytes seed almost exclusively into the popliteal lymph node (Ford, et al.,1970). Here a GVHR is mounted against F_1 lymphocytes in the lymph node and the parental cells transform, secrete a variety of immunopotent substances such as lymphokines (section 1.6) and proliferate. Amongst the substances secreted by these activated T-cells will be various mitogenic factors which stimulate proliferation of the host lymphocytes in a non-specific manner. Thus a massive proliferation of lymphocytes occurs within the lymph node. The node incorporates greater amounts of ^{3}HTdR or ^{125}IUdR and can increase in fresh wet weight from 6mg to 60mg in about 7 days. Most of the increase in weight is due to the non-specific proliferation of host lymphocytes (Grebe & Streilein,1976a,b). However, since their proliferation depends entirely upon products from the activated grafted lymphocytes, the assay is an effective measure of the reactivity of the graft cells to alloantigens.

The strains used for the assay were DA and PVG/c which when crossed gave a (PVG/c x DA) F_1 population. To stimulate a GVHR, cells from PVG/c (parental strain) were injected into the (PVG/c x DA), F_1 strain and 150-200g male animals were used throughout. The spleens from 3-4 PVG/c rats were removed asceptically and a suspension of erythrocyte-free splenic lymphocytes prepared as described previously. The viability of the cells was assessed by the trypan blue exlusion test and the concentration adjusted to 9×10^7 live cells per ml. From this stock, two further suspensions of 3×10^7 and 1×10^7 cells per ml. were prepared. The recipient

animals, of F_1 strain, were injected in the foot pad with 0.1ml of one of the stock cell suspensions. The incision was made at the heel-end of the foot and the cells injected sub-cutaneously under the loose skin at the base of the phalanges. The contralateral footpad was injected at the same time with the same dose of syngeneic lymphocytes. Following an injection of $9x10^6$ lymphocytes into the right foot pad the right popliteal lymph node became enlarged. The maximum size was reached by day 7 and was sustained for several days (fig.2.28). Day 7 after challenge was chosen as a convenient day to obtain all subsequent results. Thus after 7 days the F_1 animals were lightly anaesthetized, injected in the footpads with 0.1ml of 0.5% Evans Blue in saline, sacrificed 10 minutes later and their popliteal lymph nodes removed. The nodes were carefully cleared of all connective tissue and fat and weighed accurate to $\pm$ 0.1mg. Injections of syngeneic lymphocytes or of saline caused a negligable increase in weight while lymphocytes from PVG/c resulted in a dose-dependent increase in lymph node weight over the dose range 0.33- $27x10^6$ lymphocytes per rat (fig.2.28). The doses 9,3 and $1x10^6$ cells were chosen routinely for unprimed animals using at least four animals for each dose. The contralateral popliteal node acted as control. As the dose of cells increases there is an increased risk that cells injected into one footpad will not seed exclusively to the corresponding popliteal lymph node. If this occurs to any extent then cells will find their way to the contralateral node and react at that site. This may give undesirably high contralateral (control) lymph node weights. Figure 2.29 shows that no response was detectable in the contralateral popliteal lymph node following injection of up to $9x10^6$ cells into a foot pad.

The assay was performed in several different ways in order to test different aspects of the response (figs.2.30.,2.31.,2.32).

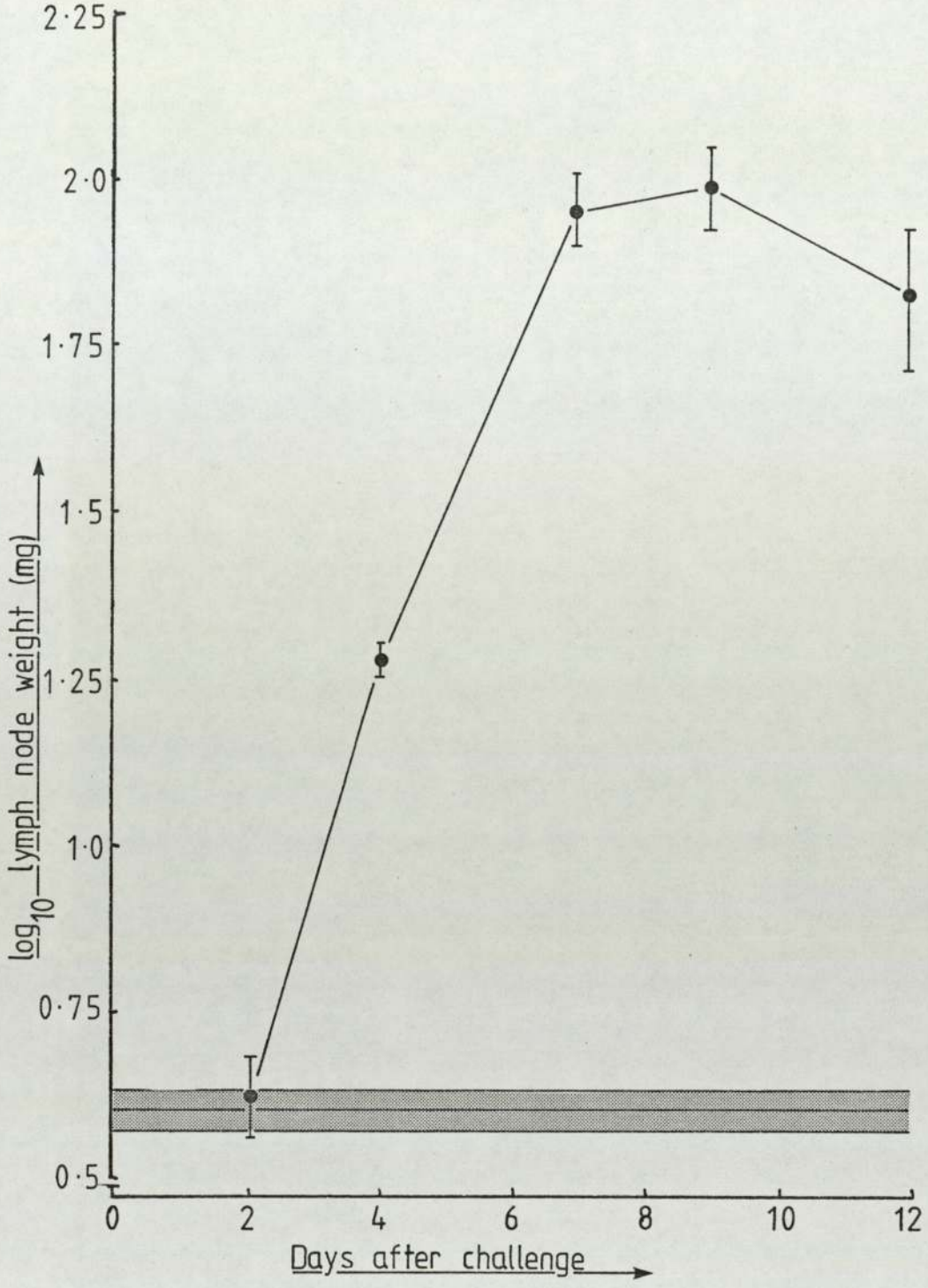


Figure 2.28. The response of popliteal lymph nodes of PVG/C x DA rats F_1 following a single foot pad injection at day 0 of $9x10^6$ lymphcytes pooled from PVG/C donors (P). The P lymphocytes recognize F_1 cells as foreign but the F_1 lymphocytes are unable to reciprocate. Thus a graft versus host response (GVHR) occurs resulting in massive hyperplasia of the host's popliteal lymph node. Hyperplasia reaches a maximum by day 7 following injection and the size of the node begins to shrink after day 9. Values are mean ± 1 S.E.M. of 4 animals in each case.

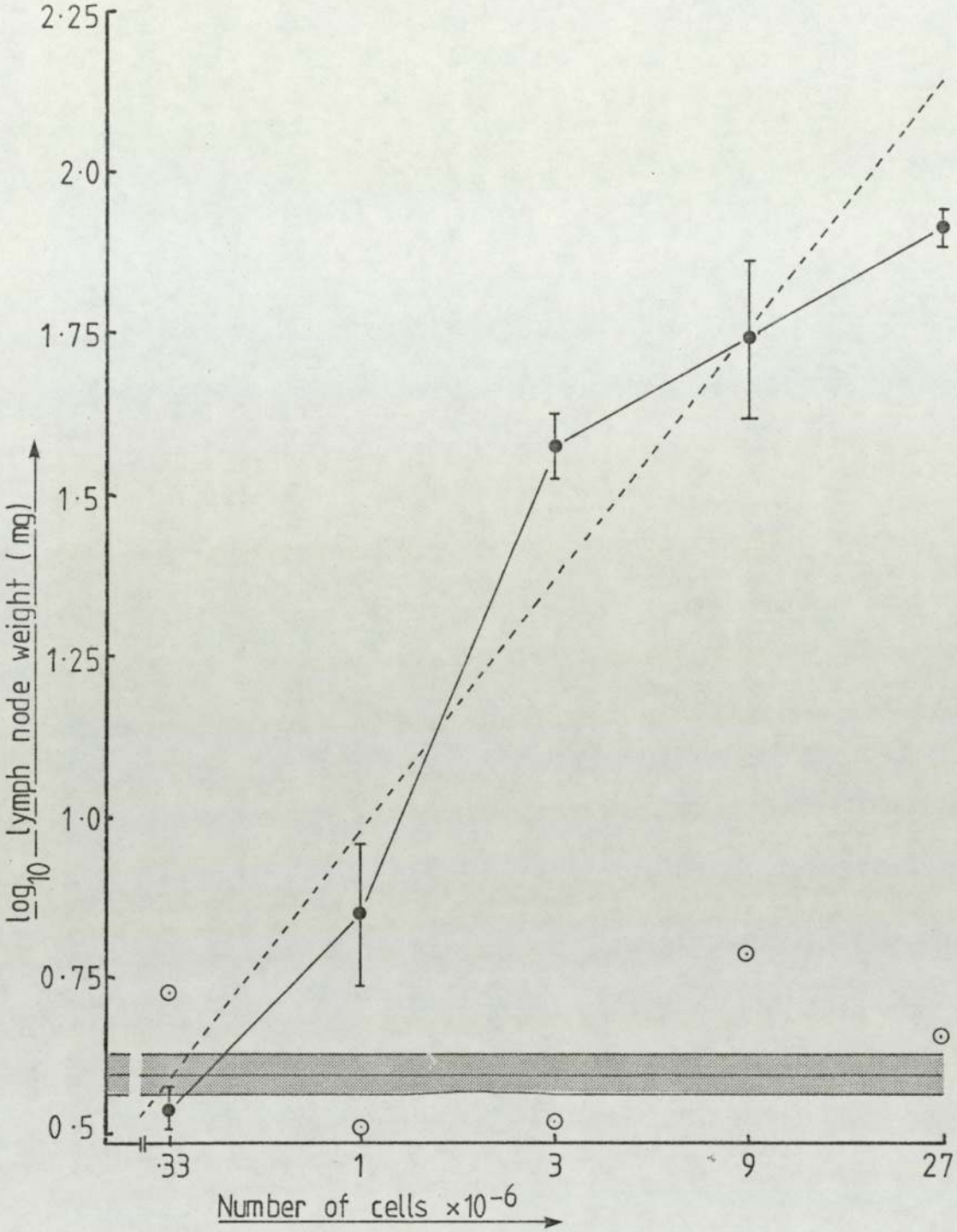


Figure 2.29. The relationship between dose injected P cells and popliteal lymph node enlargement in F_1 strain recipients. The increase in lymph node weight was linear over the range 0.33 - 27×10^6 cells. Points and aerobars are mean ± 1 S.E.M. of 4 animals and the broken line was fitted by linear regression (r[reg] = 0.98). The horizontal line and shaded area represents mean ± 1 S.E.M. of 20 contralateral lymph nodes.(⊙)

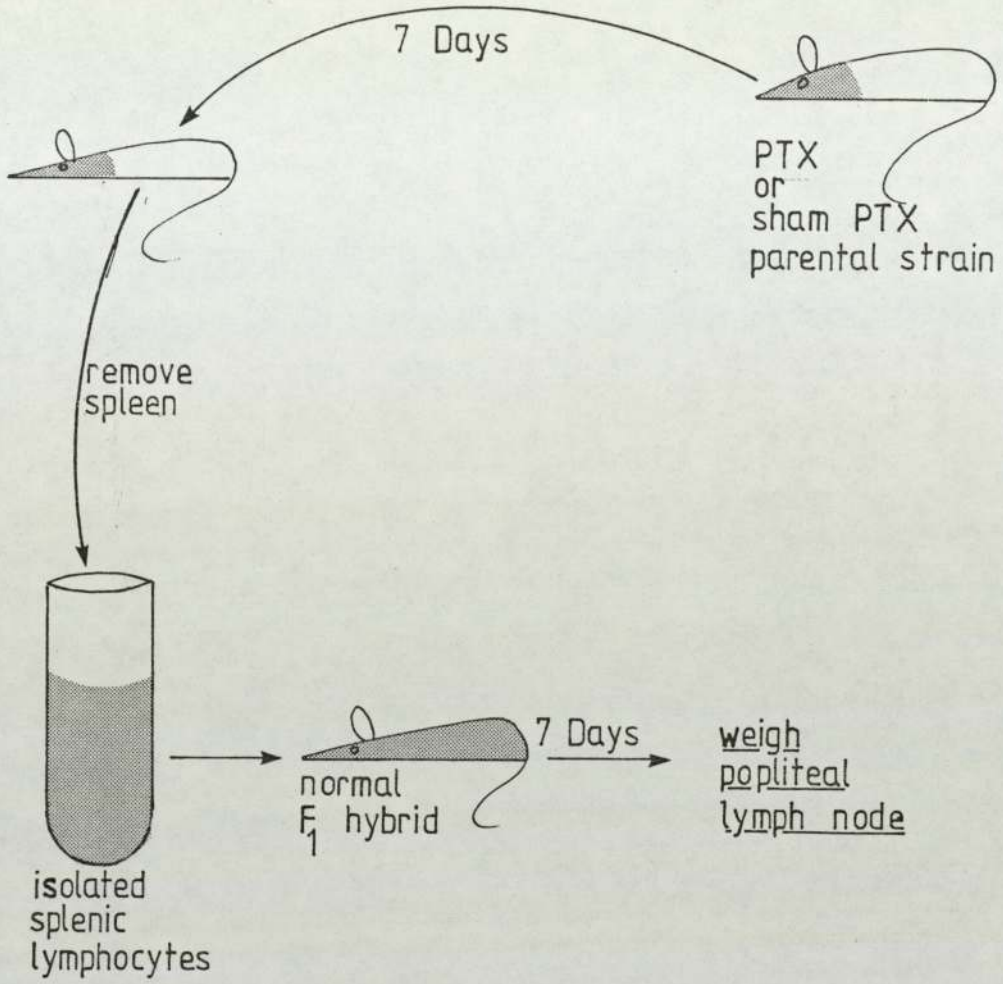


Figure 2.30. The popliteal lymph node assay used to measure the ability of lymphocytes from PTX or sham PTX donors (PVG/c) to mount a GVHR in normal F_1 recipients (PVG/c x DA). Splenic lymphocytes were obtained from rats which had recovered from the operation and injected in various doses into the footpads of F_1 hybrid rats. The degree of hyperplasia was estimated by the wet weight of the lymph node 7 days later.

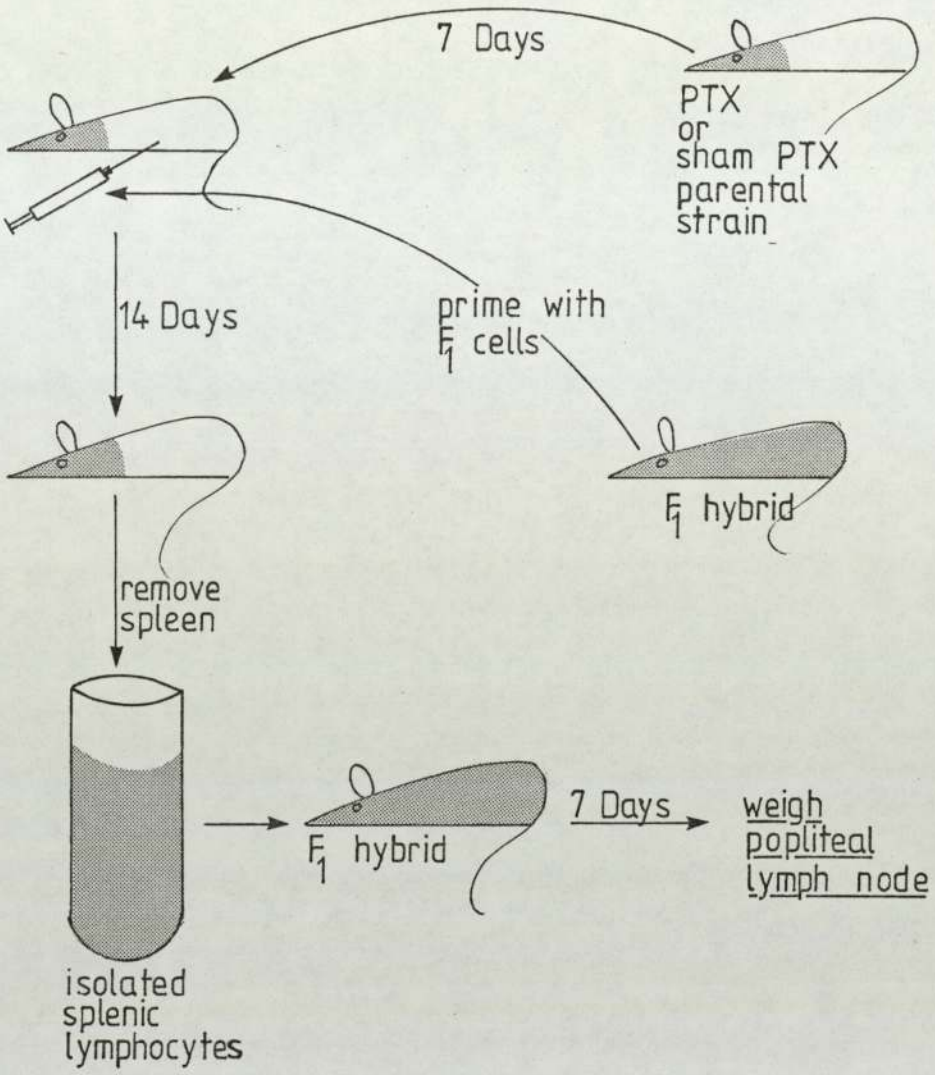


Figure 2.31. The popliteal lymph node assay used to measure the ability of PTX or sham PTX rats to develop a normal pool of memory cells. Parental strain (PVG/c) aparathyroid or sham-operated rats were challenged with PVG/c x DA) F_1 splenic lymphocytes and after 14 days a sample of parental splenic lymphocytes was injected into the footpad of normal F_1 hybrid recipients. A population of cells from primed rats will produce a greater hyperplasia than cells from naive rats.

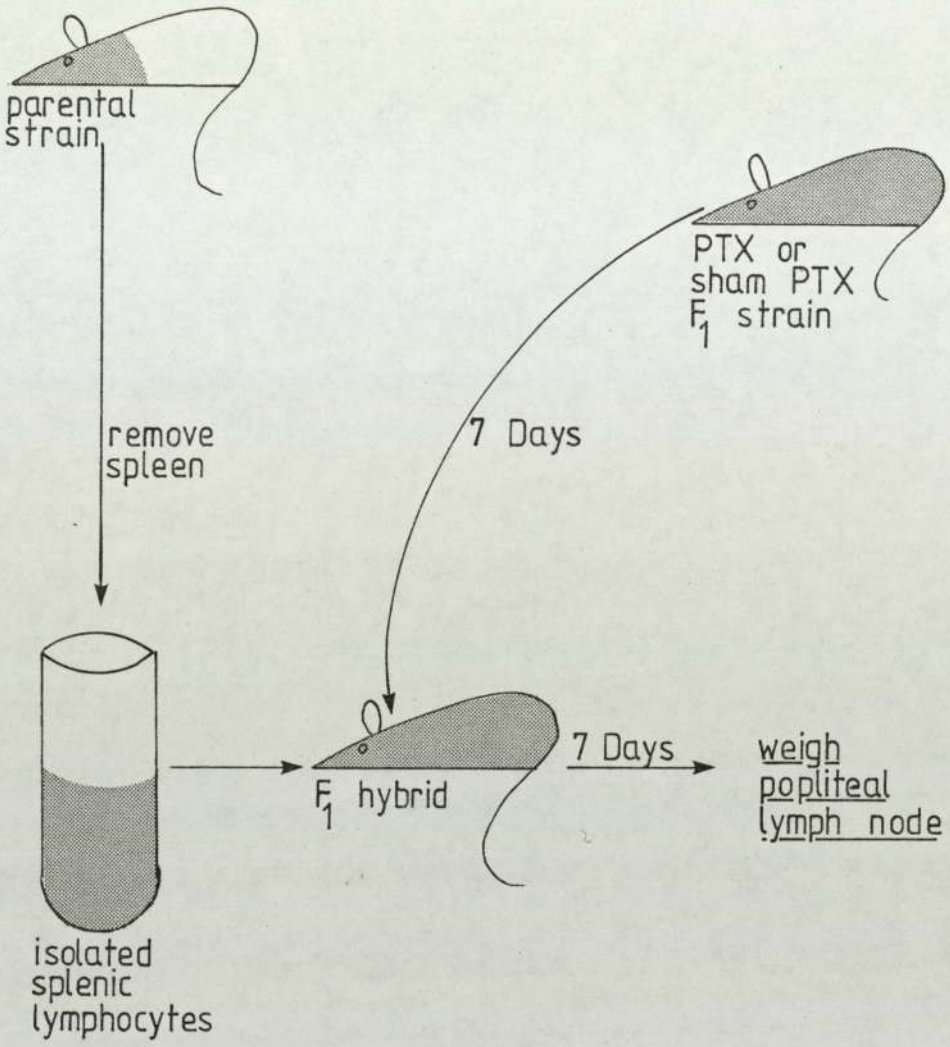


Figure 2.32. The effect of PTX of the host rat (PVG/c x DA) on the GVHR induced in the popliteal lymph node following an injection of normal parental strain (PVG/c) lymphocytes into the footpad. The rats were allowed 7 days to recover from the operation and the popliteal hyperplasia was assessed 7 days after the transfer of lymphocytes.

By transferring lymphocytes from a PTX animal into normal recipients (fig.2.30). The number of effector T-cells in the donor population can be assessed. Thus if a selective depletion of T_E- cells takes place in aparathyroid animals, a larger number of cells would have to be injected to elicit a given enlargement of the node. This would also apply if either a decrease in T_H -cells occurred or an increase in T_S cells or their relative proportion changed so that T_S: T_H increased.

Secondly, by priming the PTX parental strain rat to F_1 cells prior to the transfer (fig.2.31) a swollen pool of memory T-cells was created. If the proliferative response to the challenge of F_1 cells had been inadequate then it might be predicted that a diminished pool of memory cells would be formed. This would again manifest itself in the requirement for relatively more cells from PTX rats compared with sham PTX being required for a standard response. Finally cells from normal P strain rats were transferred to aparathyroid F_1 recepients (fig.2.32). This was to test the requirement for normocalcaemia during the stimulation of non-specific proliferation of F_1 lymphocytes by products secreted from activated P-strain lymphocytes.

Lymphocytes from sham PTX animals were compared with those from PTX animals to measure the ability of these cells to mount a GVHR. The result (fig.2.33) show that there was no difference between the response elicited by the lymphocytes from these two different sources. Furthermore, the dose/response lines for the two populations of cells are parallel. This indicates that the populations were probably of the same consistency i.e. containing equal numbers of effector cells in equal proportion to one another. These results suggest that when stimulated by allogeneic surface antigens, lymphocytes from the spleens of aparathyroid animals are

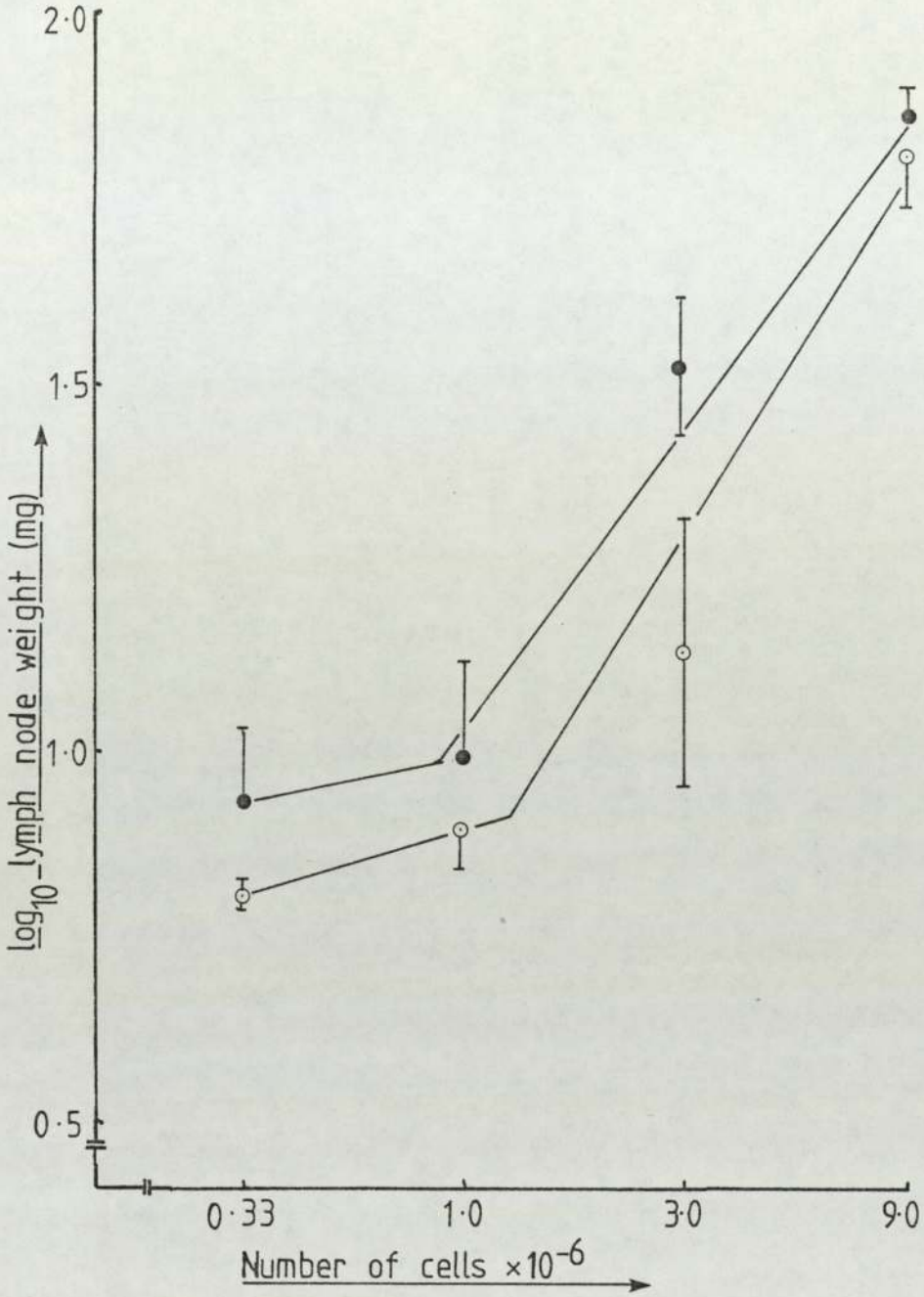


Figure 2.33. The hyperplasia of the popliteal lymph node caused by an injection of lymphocytes either from sham PTX (●-●) or PTX (o-o) donors. There is no significant difference between the response elicited by cells from either donor. Values are means ± 1 S.E.M. of 4 animals in each case and the lines were fitted by linear regression (least squares) through the responding points.

equally able to secrete the substances which elicit a non-specific proliferative response from the host node, for example lymphokines like mitogenic factor.

The results obtained with cells from primed animals (fig.2.34) indicate that PTX had no detectable effect on the development of a normal pool of memory cells. Thus the same number of cells were required to cause a standard response whether they came from PTX rats or sham PTX. Although the lines are inseparable statistically, the divergence of the PTX response from the sham PTX may be indicative of a slightly different population of cells. Such a result could, for example, be obtained by a reduction in the proportion of T_S -cells.

The reverse experiment, that is injecting lymphocytes from normal PVG/c into aparathyroid F_1, was also performed. The results (fig.2.35) show firstly that PTX did not affect the basal weight of the contralateral (uninjected side) popliteal lymph node. Secondly, for any given dose a larger number of lymphocytes had to be injected into aparathyroid recipients to elicit the same response as in normal animals. The responses of antigenically stimulated lymphocytes does not appear to be seriously affected by a lowered plasma calcium (fig.2.18-2.22) at least during humoral responses. If this applies to CMI responses, it would be reasonable to assume that the P strain lymphocytes responded normally to the antigenic challenge which they received, even though the ambient calcium concentration was low. If this was so then the non-specific stimulus provided by these P strain cells to the F_1 lymphocytes should have been the same in both PTX and sham PTX groups. It would thus appear from these results that it is the lymphocytes of the aparathyroid F_1 strain which were displaying a reduced response to the non-specific stimulus provided by the substances secreted from the PVG/c

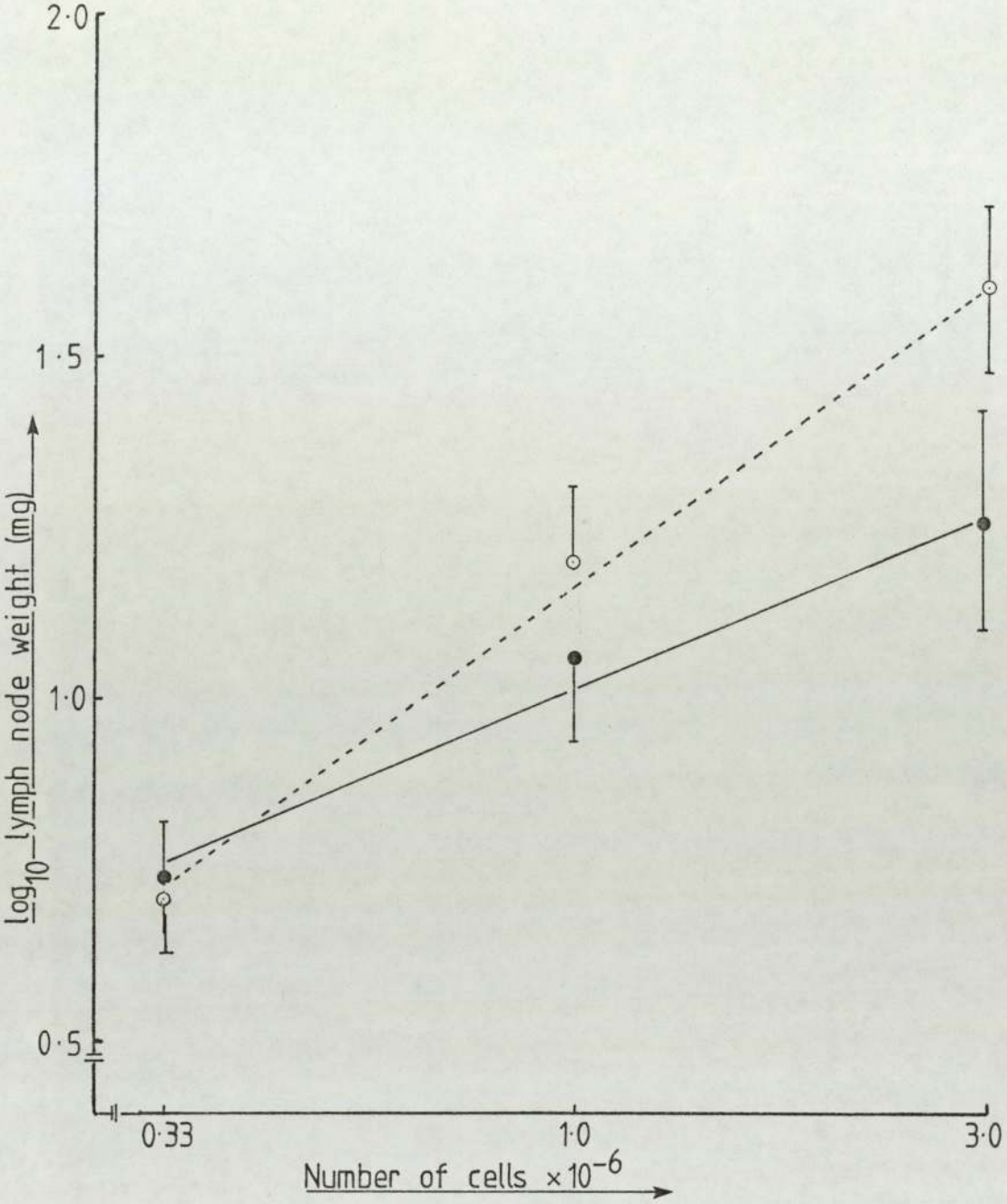


Figure 2.34. The development of memory cells in PTX and sham PTX rats. P_1 strain rats were given a single injection of 10^7 F_1 strain cells i.p. two weeks prior to injecting samples of their splenic lymphocytes into the footpads of parental strain rats. The lymph node hyperplasia which results in the F_1 strain rats was not significantly different between sham PTX (●—●) or PTX (o---o) donors. This indicates that following priming, equivalent pools of memory cells have developed in both sham PTX and PTX donors. Values are means ± 1 S.E.M. of 4 animals in each case with straight lines fitted by linear regression.

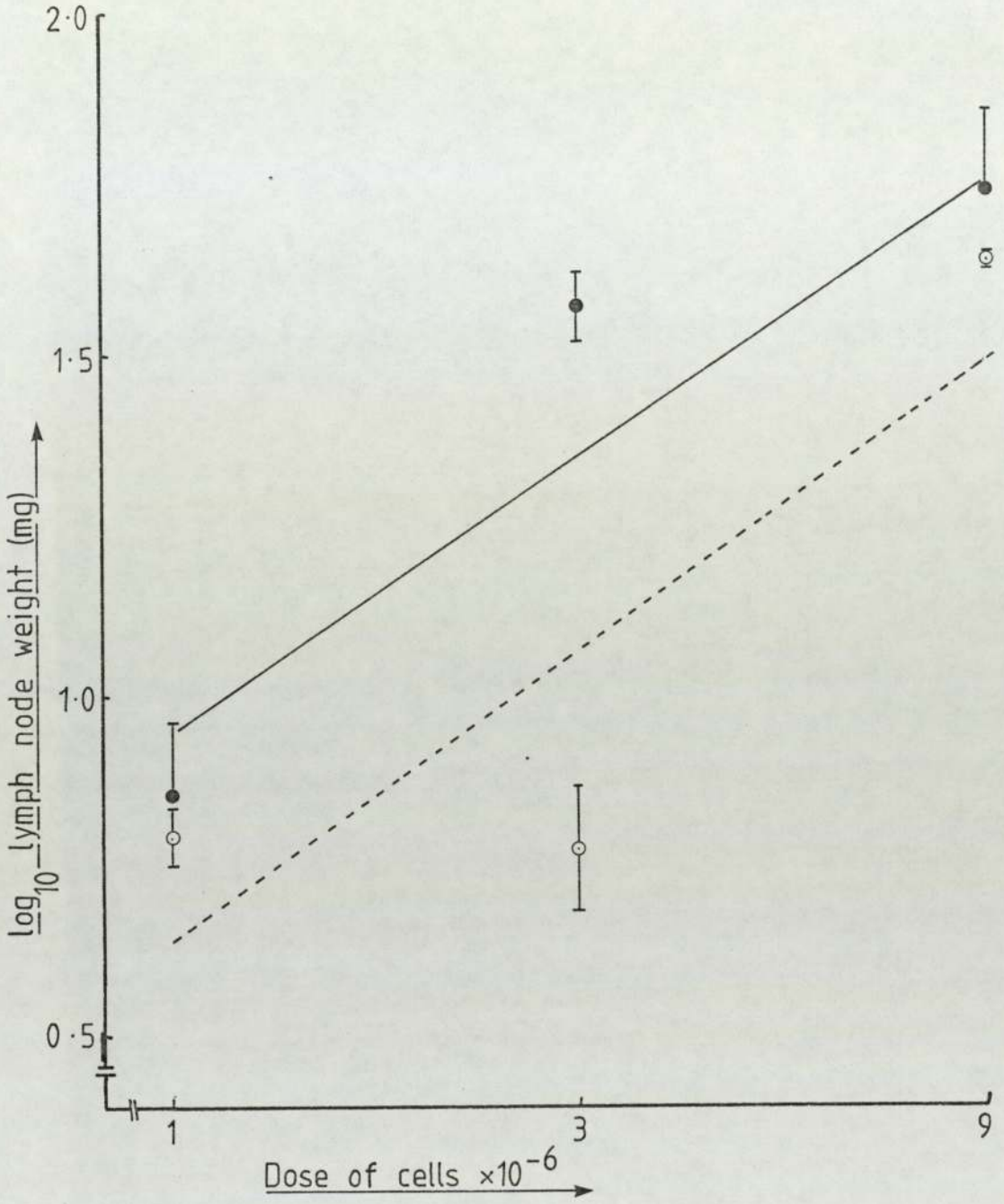


Figure 2.35. The effect of parathyroidectomy of F_1 strain rats on lymph node hyperplasia in a GVHR to P lymphocytes. Cells from a common pool of P donors were injected at various concentrations into either sham PTX (●—●) or PTX (o---o) F_1 strain recipients. In PTX recipients almost three times as many cells were required to obtain a standard response. Values are means ± 1 S.E.M. of 4 animals in each case and the straight lines were fitted by linear regression.

lymphocytes. This result could be obtained in one of two ways. Firstly all the F_1 lymphocytes which are able to respond by activation, transformation and division, may do so but undergo less rounds of division. It should be remembered that if all the lymphocytes which undergo division following challenge perform just one less division, then the result is a proliferative response of half the usual magnitude. This obvious statement can often be overlooked but illustrates how apparently small changes can invoke profound effects on the outcome of the response. The second way in which the observed result might be obtained is if a sub-population of cells failed to respond entirely or only responded poorly while the remaining population was activated normally. Although the antibody response of F_1 rats to PVG/c cells was not measured, the previous results, obtained with SRBC, indicated that the primary humoral response was not affected by PTX nor was the numbers of antibody-forming cells. It is important to remember that the proliferation of these cells was not antigen-driven but rather was a response to factors produced by activated allogeneic lymphocytes.

It is also conceivable that the secretion of mitogenic lymphokines by the parental-strain lymphocytes was impaired by transfer into a hypocalcaemic rat. The results presented in this section suggest that parathyroidectomy and the consequent hypocalcaemia have a definate, though not dramatic, effect on the immune response of rats. The operation appears to only marginally affect the humoral immune response to SRBC. Thus while there is a tendency for both the number of plaque forming cells and antibody titres to be lower these differences are not statistically significant. The observation that parathyroidectomy potentiates the DH response to oxazolone suggests an effect on a suppressive factor which may be cellular (e.g. T_S-cell or macrophage) or humoral

(a thymic factor ?). The prolongation of xenograft survival following PTX also suggests impairment of CMI as does the observation that lymph node enlargement is reduced in aparathyroid recipients. These results will be considered together with the results presented in section 2.1 and 2.2 in the discussion which follows.

Section 3.

Discussion.

3. A Discussion of the Results and Their Implications.

The proliferation of lymphocytes following antigenic stimulation is a good example of biological amplification. The number of B-lymphocytes able to produce specific IgM and IgG is initially very low but this pool of cells becomes greatly expanded, perhaps by several thousandfold, during the rounds of division which precede secretion of immunoglobulin. T-lymphocytes, with surface receptors specific for antigenic epitopes, also divide and differentiate but are totally unable to secrete immunoglobulin. Rather, their role is to provide the B-cell with an extra stimulus since B-cells can only rarely be activated by antigen alone. This T-cell stimulus by which it 'helps' the B-cell may take the form of direct cell contact or humoral factors (see section 1.7). Many of the humoral factors (lymphokines) produced are quite non-specific in their mitogenicity for lymphocytes and may activate cells which happen to be in close proximity. Lymphokines tend to be local in action however, which means that a microclimate around the antigen is conducive for mitosis. It follows that the majority of B-cells stimulated are also antigen bound. Thus non-specific stimulation is largely avoided. Where cell to cell contact is required, for B-cell activation the chance of non-specific stimulation is reduced even further. In addition to T-helper cells (T_H) some T-cells may secrete substances which inhibit B-cell activation either directly or indirectly and are called T-suppressor cells (T_S). The ratio of T_H to T_S, and therefore whether antigenic stimulation results in antibody production or not, depends on several factors not all of which are fully understood. Certainly the antigen dose plays a part and the ratio of T_H to T_S probably also varies throughout the course of a normal response. Thus T_H is probably maximal at the early stages and T_S maximal as the response declines.

The proliferation of cells in the immune response thus serves two purposes. Firstly to expand a clone of specific effector cells. (While antibody producing cells have been described above, the effector cell might just as easily be a cytotoxic T-cell). Secondly, and no less important, the proliferation marks the expansion of clones of regulatory cells. In addition to these proliferative events, which are antigen driven, there can also be non-specific stimulation by T-cell products.

This outlines the reality behind the clonal selection theory (Burnet 1959) which is still basically acceptable despite the need to superimpose a myriad of intercellular control mechanisms on the clonal expansion. However, one essential requirement for clonal expansion of this type is that the cell must possess an antigenic receptor. At first sight the proliferative response which was observed in bone marrow following antigenic challenge was indicative of antigen sensitivity. The proliferation of the bone marrow did not occur following administration of isologous erythrocytes or saline so that it appeared to be an immunological phenomena. Elevated proliferative activity in lymphoid elements was likewise observed in the spleen (fig.2.17). These are similar to results obtained in the mouse (Harris & Pelc,1970) following SRBC when an increase in incorporation of ^{3}H thymidine was observed. However, in the mouse the bone marrow is not thought to play a significant rôle in the primary immune response to sheep erythrocytes (SRBC) (Benner, van Oudenaren & De Ruiter,1977). This is probably true for rats also and is supported by the observation that plaque forming cells are extremely scarce in the bone marrow during the primary immune response to SRBC (fig.2.33). In contrast, the spleen is clearly a major site for antibody production in the

rat and consequently many plaque forming cells (PFC) develop there during the primary response to SRBC (figs.2.22 2.23).

A further dichotomy between splenic and marrow lymphocytes was apparent when considering the susceptibility to inhibition of proliferation by manipulations of the calcium homeostatic system. It was thus observed that a marked systemic hypercalcaemia developed concomitant with the peak of proliferative activity in the bone marrow and spleen (figs. 2.1, 2.2 & 2.17). Considering the report that injections of $CaCl_2$ also causes elevated bone marrow proliferation it was possible that calcium per se might be driving the proliferative response. Parathyroidectomy prevented the development of hypercalcaemia (fig.2.12) and also prevented the development of elevated bone marrow mitosis (fig.2.11) thus supporting the hypothesis that an hypercalcaemia can stimulate such a response. If the antigen stimulus received by the bone marrow cell is weak, perhaps because of low antigen receptor density or affinity, then a low calcium environment may further reduce the stimulus below the threshold necessary for cell activation. This possibility was eliminated by the experiment with aparathyroid animals rendered normocalcaemic with high calcium/low phosphorus diet (fig.2.13). In this experiment the bone marrow failed to show a proliferative response in the presence of antigen even though the plasma calcium concentration was normal. This is strong evidence that the hypercalcaemia itself is responsible for the stimulation of bone marrow proliferation in intact animals. As a corollary to this, the cells of the bone marrow must be antigen insensitive. Despite this however, the elevated proliferative activity of the bone marrow did not occur if isologous erythrocytes were injected i.e. an antigenic stimulus was a prerequisite for

elevated bone marrow proliferation. It would seem therefore that the cells of the bone marrow were stimulated as a consequence of an immune response but not directly by antigen.

In direct contrast to these results parathyroidectomy (PTX) failed to prevent the proliferation of splenic lymphocytes in response to antigen (fig.2.17). This implies a fundamental difference in the type of lymphocytes which are responding in these two tissues. The splenic lymphocytes were almost certainly mature lymphocytes with functional antigenic receptors. They were thus capable of stimulation by antigen, and from the results obtained, continued to be stimulated in low calcium environment and in the absence of an hypercalcaemic episode. However, the cells of the bone marrow were probably immature lymphocyte precursors without antigen receptors.

At the outset of these experiments the experimental data already available suggested that calcium played a central role in lymphocyte activation (as it does) and that this might be demonstrable <u>in vivo</u> by observations using aparathyroid (hypocalcaemic) rats. If hypocalcaemia induced by parathyroidectomy inhibited lymphocyte proliferation then an anticipated result of this might be reduced clonal amplification following antigenic stimulation. A marginal effect on clonal expansion would have dramatic effects on the eventual clone size e.g. one less division would half the number of cells in the clone. It was anticipated, therefore that aparathyroid animals would be immunodeficient and experiments were initiated to test this. The findings (see 2.2) that the antigen-induced proliferation of splenic lymphocytes continued unabated in aparathyroid rats indicated that mature lymphocytes were fundamentally different from those in the bone

marrow with respect to the blockade of cell proliferation induced by hypocalcaemia. It was evident that cells which could be stimulated by antigen were not susceptible to mitotic blockade by lowering the plasma calcium. It follows from this that clonal expansion might not be expected to be reduced. However, it was not possible to ascertain whether the apparent lack of effect on the splenic lymphocytes was total or only partial. Thus it was possible that some clones of lymphocytes, particularly those with receptors with low epitope affinity may fail to respond to antigen stimulation under sub-optimal conditions e.g. in a low-calcium environment. This may manifest itself by impaired immunocompetence.

An animal can be considered to be fully immunocompetent if the following immunological features are both evident, and expressed to a normal degree:

1) The production of circulating specific antibody after antigen stimulation.
2) The capacity to reject skin xenografts.
3) The ability to develop delayed hypersensitivity.
4) Proliferation of immunocompetent cells after antigenic stimulation.
5) The capacity for immunological memory.

Aparathyroid rats were accordingly tested for the criteria of immunocompetence cited above.

The measurement of specific immunoglobulin secreted in response to immunization with SRBC revealed no significant difference between PTX and sham PTX or normal animals. The total antibody concentration and that of IgG class was slightly reduced but the results were not statistically significant (figs.2.18, 2.19, 2.20). A similar picture emerged with the number of plaque forming cells in the spleen (figs.2.23, 2.24). These two results taken together

confirm two points. Firstly that the clonal expansion of antibody precursor cells is not severely affected by parathyroidectomy and secondly that the differentiation into antibody-secreting cells and the capacity of those cells to secrete antibody is likewise not affected. Again, these results were not totally unequivocal. For example, the number of direct plaques was significantly less in PTX animals when compared to normal controls. However, the sham operation clearly had some effect (? stress) since this too reduced the number of direct plaques compared to normal controls. The overall result shows a tendency for the maximum number of plaque forming cells to be reduced in PTX animals though the time onset of the development of the humoral immune response to SRBC is unaffected.

These findings are consistent with a lack of effect of PTX on peripheral secondary lymphoid tissue. A notable feature of parathyroidectomized animals is rapid involution of the thymus which is steroid independent (Perris, et al.,1970). As pointed out earlier, if this sort of atrophy were to occur throughout the lymphoid tissues a marked immunodeficiency would be expected following PTX. Since this does not appear to be happening the situation is somewhat like an adult thymectomy (ATX) induced by withdrawl of PTH. Following ATX there are no immediate results. Mice develop lymphopoenia but only after several months (Metcalf, 1965; Miller,1965) and there is no effect on the primary immune response but the secondary response is decreased (Simpson & Cantor, 1975). It should be mentioned that during the secondary response to SRBC parathyroidectomized animals produced specific circulating antibody of both IgM and IgG classes in amounts indistinguishable from control groups (data not shown).

The above data suggests that neither the proliferation of B-cells nor of T_H-cells is affected. The remaining data on the immunological competence of aparathyroid rats was concerned with cell-mediated immunity (CMI).

In the delayed type hypersensitivity (DTH) response to oxazolone the animals were first sensitized to the chemical (see section 2.3). During this sensitization period, proliferation of T-cells occurs. Upon rechallenge the magnitude of the response is related to the degree of proliferation which occurred during sensitization. Furthermore the relative numbers of T_H:T_S will influence the response. The results showed that the response to oxazolone was not inhibited by PTX but rather, was potentiated with respect to sham PTX controls (fig.2.27). There are two possible explanations for this observation. Firstly it is possible that during the sensitization phase the proliferation of specific T_S-cells was inhibited. This would result in a reduction of T_S-cell clone which would in turn lead to an enhanced response upon rechallenge. Secondly, hypocalcaemia affects muscle tone which causes a reduced lymphatic flow (Deysine, Mader, Rosario & Mandell,1974). This may result in persistence of antigen at the site of application due to less efficient clearance and thus aggravate the response.

A skin xenograft elicits a powerful CMI response. However, this does not preclude the co-operation of antibody mediated responses and macrophage involvement so that, in fact, both these elements are involved (section 1.7). However, from the finding that humoral responses to SRBC remain unaffected by PTX it seems likely that any effect on skin graft rejection is due to the cell--mediated axis of the response. The lymph node assay (Ford,1970)

for graft versus host response (GVHR) was chosen as a alternative to the skin graft rejection for an assay of CMI because of its greater sensitivity and versatility. As detailed in section 2.3, a GVHR develops in the popliteal lymph node of (PVG/c x DA) F_1 rats after splenic lymphocytes from PVG/c rats (P) are injected into the footpad. Although P strain cells recognize F_1 cells in the lymph node as foreign and respond accordingly, the F_1 cells cannot reciprocate. However, in the confines of the lymph node much non--specific stimulation of F_1 cells occurs by products secreted from P lymphocytes. Thus lymph node hyperplasia is largely caused by proliferation of host (F_1) lymphocytes (Grebe & Streilein,1976a,b). By using this assay it was possible to test three features of the immune response in parathyroidectomized rats.

1) The number of cells in a population of splenic lymphocytes from PTX rats which were able to recognize and respond to alloantigens.

2) The ability of PTX rats to develop a normal pool of memory effector cells.

3) The ability of PTX rats to respond to non-specific mitogenic stimuli from donor lymphocytes by lymphoproliferation.

The first point is really testing whether peripheral lymphocytes are as susceptible to the withdrawal of PTH as are those of the thymus. If so, a reduced number of T-cells may be expected in the splenic population. The results (fig.2.33) clearly eliminate this possibility. The finding that the population of splenic lymphocytes from primed PTX rats (fig.2.34) also contains equal numbers of memory cells clearly demonstrated that a normal clonal expansion had occurred in the primary response. These results support the earlier finding that antigen-sensitive cells seem to be unaffected by PTX (section 2.2. and figs. 2.17).

In contrast to these results was the finding that about three times as many normal splenic lymphocytes were required to elicit a standard response in PTX rats than in control groups (fig.2.35). This indicates that the ability of the F_1 strain lymphocytes in the PTX host was less able to respond to the non-specific stimulus provided by the products from responding P strain cells. The result is consistent with the notion that non-antigenic mitogenic stimuli may require the presence of a functional parathyroid to be effective

As detailed in section 1.4, many physiological proliferative events occurring in the bone marrow and thymus are associated with a hypercalcaemic episode. Thus the elevated bone marrow proliferation which occurs following haemorrhage (Perris et al.,1971) or injections of cobaltous chloride (Perris,1971) during pregnancy (Perris,1971) and in the oestrus cycle (Hunt & Perris,1974; Smith & Perris,1976) are all associated with hypercalcaemia. The result obtained here (figs. 2.1-2.6) follows this general pattern and further confirms a relationship between plasma calcium concentration on the one hand and bone marrow mitotic activity on the other. The finding that these two phenomena occur together only when an antigenic stimulus is provided, strongly suggests that the effect is mediated by antigen sensitive lymphocytes. These antigen--sensitive cells may have been in the secondary lymphoid tissue which were demonstrated to contain antigen-sensitive elements. However, the mitogenic factors produced in this case would have to be effective systemically. This seems unlikely for a putative lymphokine-like substance as these tend to be only locally active. An alternative is that a small percentage of antigen-sensitive cells were present in the bone marrow and provided a non specific mitogenic stimulus to the antigen-insensitive cells which were

adjacent. These mature cells may have either migrated back to the marrow or were ready for export to peripheral sites. Thus the hypercalcaemia observed must be directly or indirectly caused by some product of the immune response.

Bacterial lipopolysaccharide from E. coli (LPS) was also found to stimulate bone marrow mitosis and cause an elevation of plasma calcium (fig.2.6). LPS is a polyclonal B-cell activator (Greaves & Janossy,1972; Andersson, et al.,1972) and if it could be assumed that this were the only site at which LPS acts then it would strongly implicate B-cell involvement in the hypercalcaemic phase. Unfortunately LPS also stimulates macrophages (Spiznagel & Allison,1970; Edelson, Zwiebel & Cohn,1975) and, through macrophages, T-lymphocytes (Gery, Gershon & Waksman 1972; Gery & Waksman,1972) which makes interpretation difficult. However, the response to LPS is very rapid, reaching a peak in 24 hours compared to three days following antigen (figs. 2.1, 2.2 & 2.6). It is most likely that at the dose used (250ug/Kg) LPS was acting in a mitogenic capacity rather than in an antigenic one. If this is so there is clearly no absolute necessity for antigenic stimulation in order to obtain a hypercalcaemia. This is perhaps not surprising since many lymphokines are obtained from PHA-stimulated cultures.

It has thus been demonstrated that the hypercalcaemic phase which follows antigenic stimulation primarily affects antigen insensitive lymphocytes. During the immune response cells divide and differentiate to plasma cells or effector T-cells. These cell types are end cells and if left unchecked the constant flow of cells into an active effector cell compartment would gradually deplete the virgin lymphocyte pool. If however, some product could cause the stimulation of lymphocyte precursors into a transitory burst of

elevated cell proliferation then the cell pool size might remain constant but with a swollen pool of specific memory cells. Thus the hypercalcaemia could be a manifestation of one part of a feedback loop designed to maintain cellular homeostasis. Clearly intact parathyroid glands are mandatory for the completion of this loop but whether there is an actual increased output of PTH following challenge is impossible to say from the data presented. That question will have to await a reliable and sensitive assay for rat PTH. It seems unlikely however, that a product of an immune response could stimulate PTH secretion. More likely is that the site of action is the bone itself and that the presence of PTH is required as a synergist. This mode of action would be quite similar to that proposed for prostaglandin- and vitamin D- induced bone resorption. The main features of this model are embodied in figure 3.1.

The results presented here clearly show that the parathyroids have an effect on the tissues of the immune system. The effect of withdrawl of PTH on primary immune responses is not great, though there are clear indications of immune impairment. The effect of plasma calcium on the bone marrow opens up exciting possibilities in terms of the relationship between the endocrine and immune systems. It tends to be generally accepted that the two must be mutually influential though relatively little work has been performed in this area. The interrelationships of lymphocytes and hormones is likely to be complex and perhaps quite subtle but an understanding of these mechanisms is fundamental to our appreciation of the immune response as an integrated physiological phenomenon.

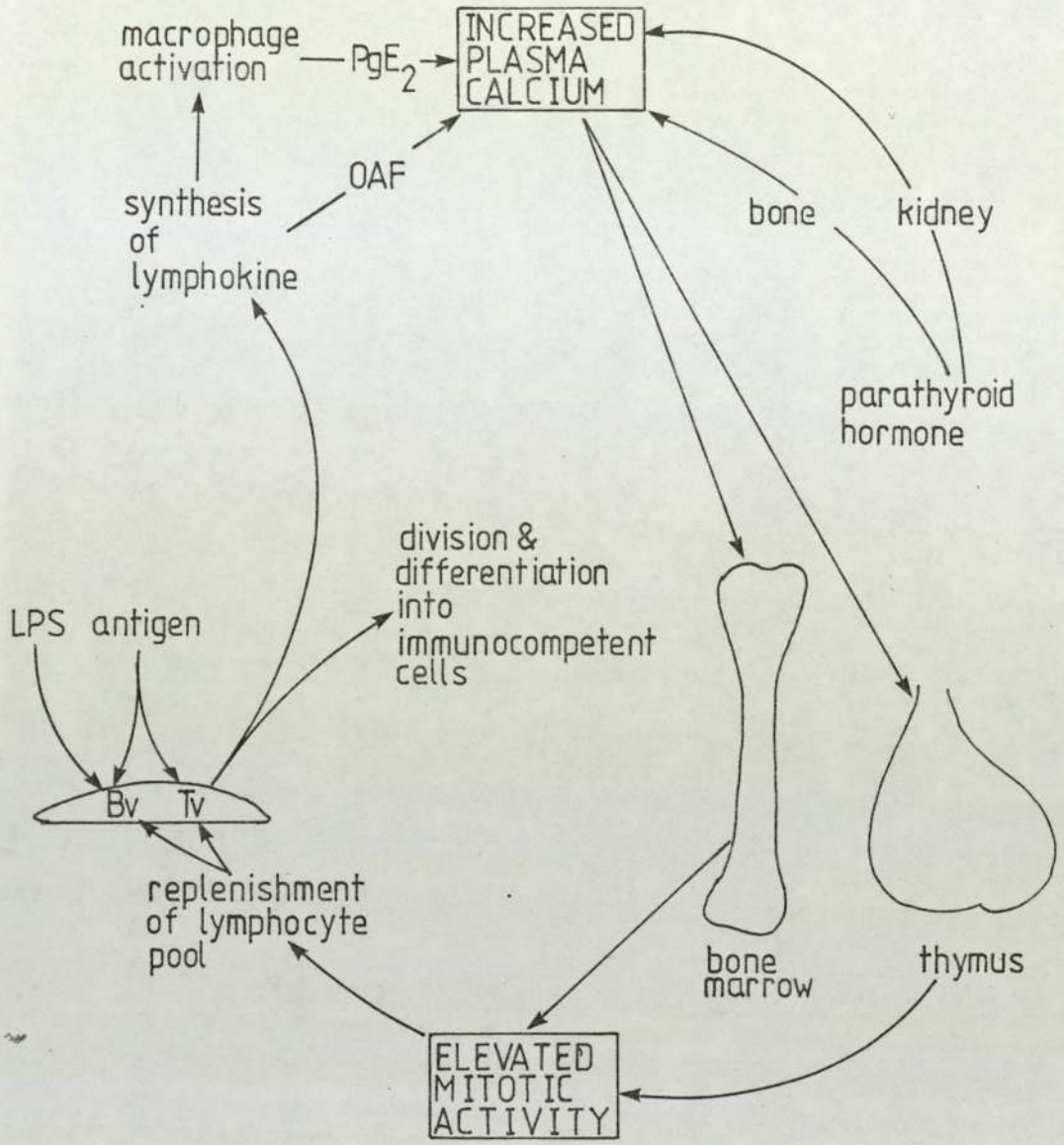


Figure 3.1. A schematic diagram which collates the results obtained into a working hypothesis. Antigenic or mitogenic stimulation causes cell division and differentiation into immunocompetent cells. Some of the products released during this process are known to cause bone resorption e.g. prostaglandins and osteoclast activating factor (OAF). The putative substance responsible for the observed hypercalcaemia following antigenic challenge probably requires the presence of parathyroid hormone to be effective. Elevated plasma calcium results in a non-specific stimulation of division and differentiation of bone marrow and thymus cells, and the daughter cells from these divisions may serve to replenish the lymphocyte pool. Thus antigen stimulation elicits a response which aims to reconstitute the lymphoid cell populations.

Section 4.

Appendix.

4.1 The Use of Radiolabelled Nucleotides for the Measurement of DNA Synthesis.

The incorporation of radiolabelled nucleotides into DNA has become the most commonly used method for the assay of DNA synthesis. Its main advantages are the speed and reproducibility of sample handling made possible by automatic radioactivity counting devices.

The method originally adopted for the estimation of DNA synthesis in the spleen and bone marrow was the incorporation of a radiolabelled DNA precursor, iodinated (^{125}I) deoxyuridine (^{125}IUDR). Since ^{125}I is a gamma-emitter, it can be detected using a gamma counter so that no special sample preparation is required other than the removal of unbound isotope. This method has been used for the estimation of DNA synthesis in lymph nodes during oxazolone-induced hyperplasia (Pritchard & Micklem,1972).

The experiment was first attempted *in vivo* where 20μCi/200g rat had been found by a preliminary experiment to give adequate incorporation in the tissues of interest. Rats were given 20μCi of ^{125}IUDR i.p. in saline and sacrificed at intervals thereafter. The level of isotope in the plasma rose rapidly following administration and then fell as the isotope became incorporated in the tissues (fig. 4.1). Corresponding to this pattern a rise in the amount of isotope incorporated in the tissues was observed which reached a plateau by 2 hours (fig. 4.1).

The bone marrow was removed by aspiration (see section 2.1) fixed for 30 minutes with 10% neutral formol saline and washed 4 times with 70% ethanol, centrifuging at 800xg between washes. This procedure removes 98-99% of unbound ^{125}IUDR (Pritchard & Micklem,1972). The pellet was then counted until at least 10,000 counts had been accumulated on a gamma counter. Spleens were fixed

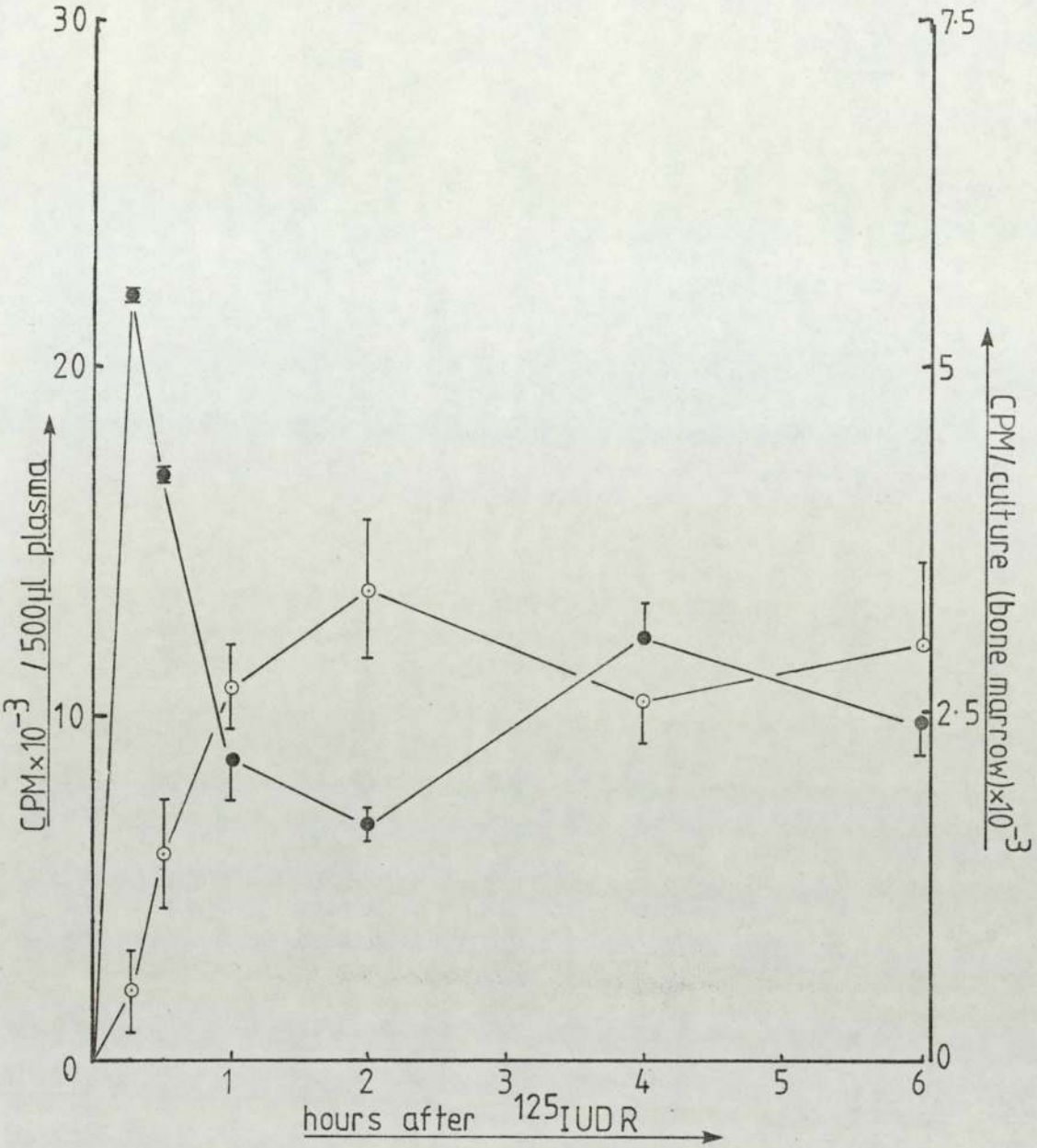


Figure 4.1. The level of ^{125}I activity in the plasma (●—●) and the bone marrow (⊙—⊙) following a single injection of 20 µCi of ^{125}IUDR i.p. in saline at time 0. The isotope appears very rapidly in the serum but most becomes incorporated into the tissues within the first two hours. This is reflected by the levels detectable in bone marrow over the same period.

for 24 hours in 10% neutral formol saline and washed 4 times with 70% ethanol allowing 24 hours between washes.

The change in the amount of incorporation of ^{125}IUDR was measured in spleen and bone marrow following antigenic challenge with 10% saline-washed sheep erythrocytes (SRBC). The results (fig.4.2) show that unfortunately the increase in amount of isotope incorporated was minimal. Thus in bone marrow a slight rise was observed on day 2 following SRBC while in spleen, there appeared to be a biphasic pattern (fig.4.2). In view of these results it was felt that this method was unlikely to prove a useful assay for DNA synthesis after antigenic challenge.

As an improved method cell suspensions were prepared from the tissues of challenged animals on the relevant day following antigenic and incubated for 3 hours at 37°C with 1μCi of ^{125}IUDR per 200 μl microculture in microtitre plates (Cooke). After this period they were harvested onto glass fibre filter mats (Flow Laboratories), washed with water, dried and counted by gamma counting as before. This procedure gave essentially the same results as the in vivo method.

Since ^{125}IUDR is incorporated only at about one fifth the efficiency of tritiated thymidine ^{3}HTdR, it was decided to use this nucleotide as an alternative to deoxyuridine. The weak β-emission from tritium was detected by liquid scintillation counting. Cell suspensions were prepared in RPMI 1640 medium buffered with 20mM HEPES and supplemented with 5% foetal calf serum. Microcultures containing 10^6 cells in 200 μl were incubated for 3 hours at 37°C in the presence of 1μCi ^{3}HTdR (specific activity; 2Ci/mM) by which time maximum uptake of the isotope had been achieved (fig.2.15). After this time they were harvested onto glass fibre filter mats as before washed with water and air-dried at 60°C.

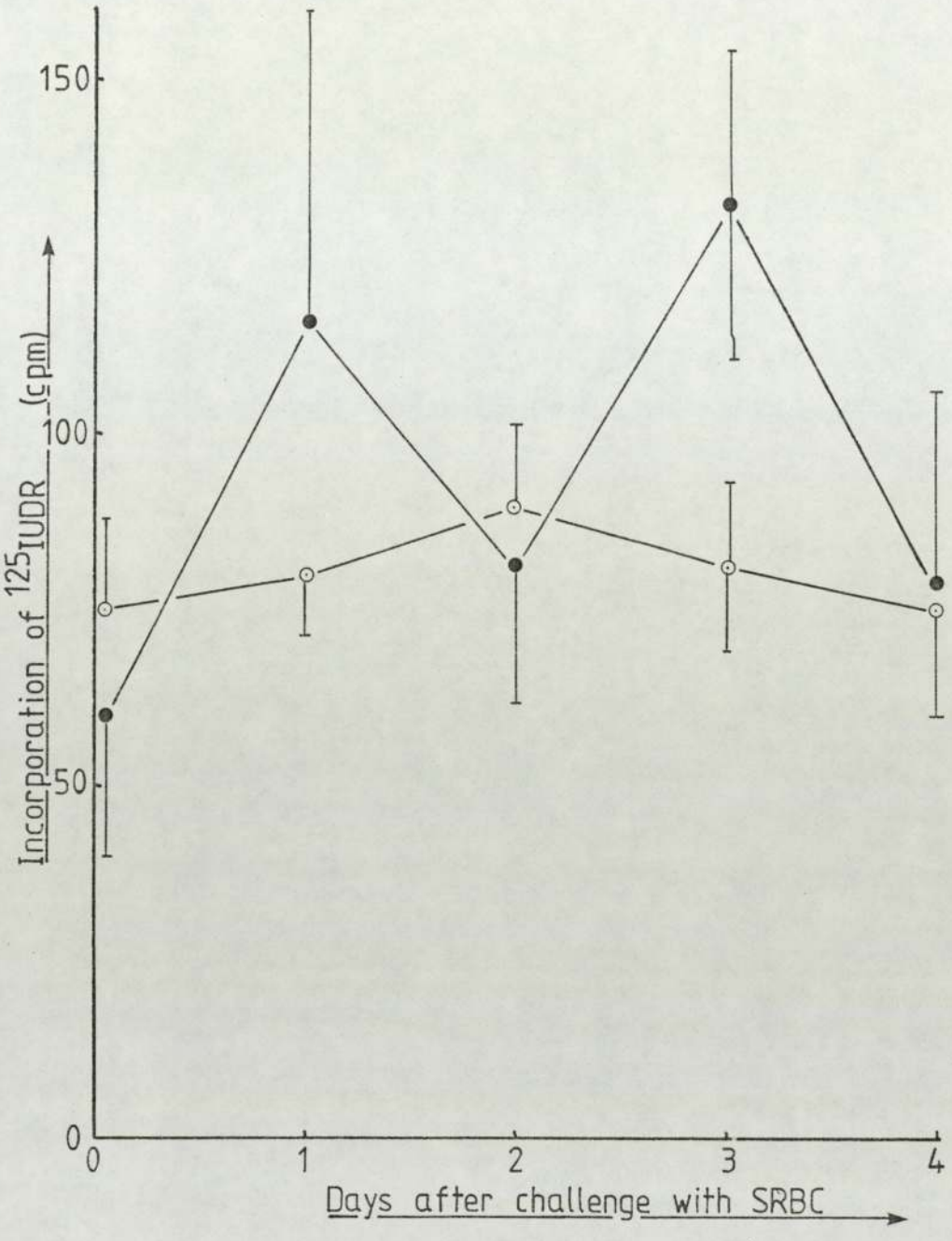


Figure 4.2. The incorporation of radiolabelled deoxyuridine (^{125}IUDR) into the spleens (●—●) and bone marrow (o—o) of normal rats following a challenge with 1ml of 10% SRBC in saline each rat received 20 μCi ^{125}IUDR i.p. in saline 2 hours before sacrifice. The incorporation into the spleen, represented as CPM/g tissue, was erratic with large variation in individual values making analysis difficult. The very slight rise in bone marrow incorporation (results expressed as CPM/10^6 cells) was insufficient for assay purposes.

This washing procedure has been shown to wash any ^{3}HTdR not incorporated in DNA from the cells (Ahern,1976). After drying, the filters were punched out into a liquid scintillation vial and 0.5ml of hyamine 10x hydroxide (BDH) added. The vial was then incubated for 2 hours at 50°C. The hyamine hydroxide is strongly basic and acts as a tissue solubulizer to obtain the sample in the liquid phase prior to addition of the scintillant.A scintillation cocktail (5ml) containing 5g PPO and0.1g POPOP in 1 titre of scintillation grade toluene (BDH) was added and the samples stored in the dark overnight to allow disappearance of any chemiluminescence caused by the addition of hyamine 10x hydroxide. Slight variations in counting effeciency were detected using a standard channels ratio method (Neame & Homeward,1974) and the appropriate corrections made.

The results obtained with these methods (fig.4.3) underline the inadequacy of these standard techniques for the measurement of an increase in DNA synthesis in lymphoid tissues during the primary immune response to antigen. Haemorrhaged rats (5ml of blood removed by cardiac puncture 2 days previously) were included as a positive control and the result obtained clearly shows that the expected increase in DNA synthesis in the bone marrow was detectable using the methods described above (fig.4.3). In contrast, no increase in the rate of DNA synthesis was detected on any of the first four days following antigen challenge (data shown was obtained on day 3).

It was concluded from these findings that the incorporation of radiolabelled nucleotides into DNA, followed by either gamma or liquid scintillation counting, could not provide a sufficiently sensitive assay for DNA synthesis. The difficulties encountered were probably due to the low number of cells which are stimulated by a novel antigen (see section 1.6) since the methods described

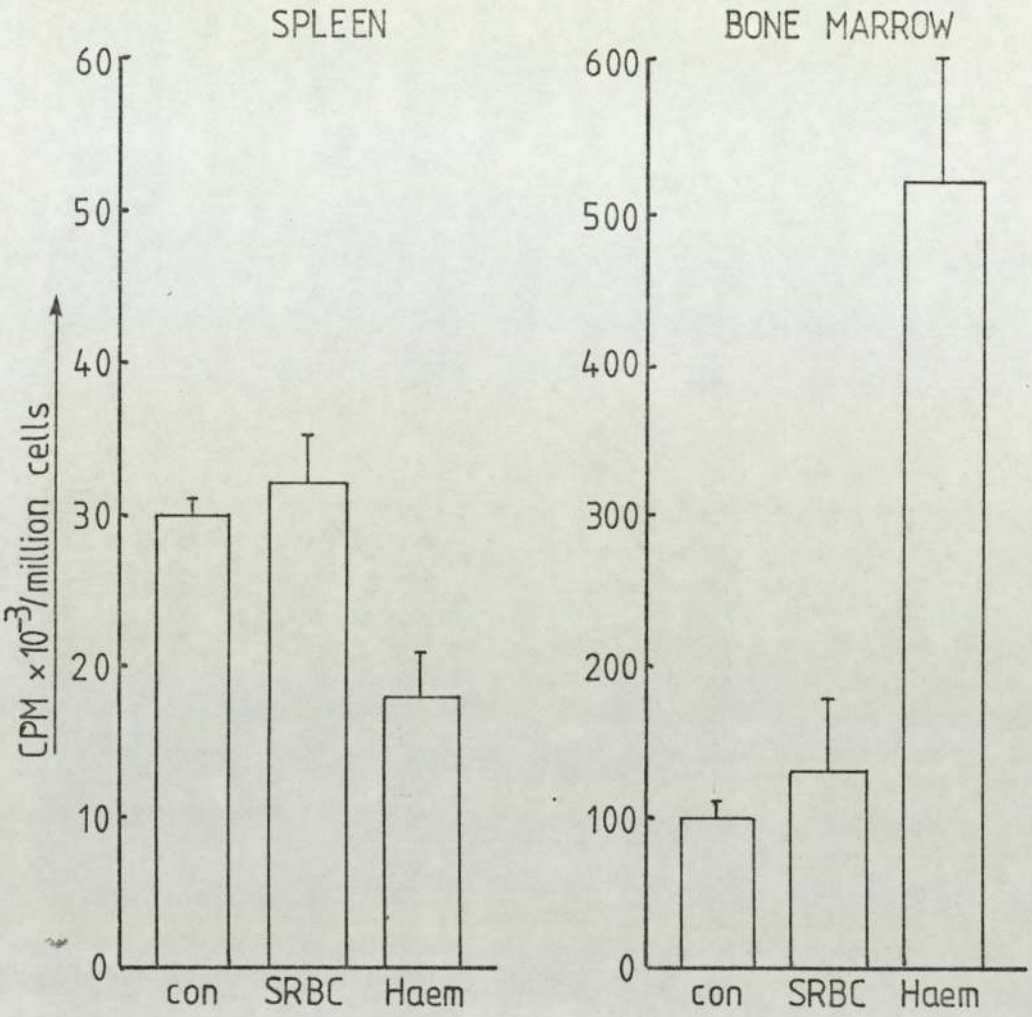


Figure 4.3. The incorporation of tritiated thymidine (^{3}HTdR) into spleen and bone marrow cells from either normal control rats (con), 3 days after 1ml of 10% sheep red blood cells (SRBC) or 2 days after haemorrhage (Haem). Cells were incubated in microculture plates for 2 hours in the presence of 1 μCi ^{3}HTdR before harvesting onto glass fibre filter mats and processing for liquid scintillation counting. Following SRBC there was no detectable increase in ^{3}HTdR incorporation into either spleen or bone marrow cells. In contrast, haemorrhage caused a marked elevation of incorporation into bone marrow as expected due to increased erythropoietic activity. This result demonstrates that correct methodology was being used for the detection of increased DNA synthesis.

are used routinely for lymphocyte transformation studies where agents such as phytohaemagglutinin cause a considerable percentage of the cell population to enter DNA synthesis. It was therefore considered necessary to use autoradiography for the detection of enhanced cell proliferation in the spleen following antigenic challenge. The data obtained by this means have been presented in section 2.2.

4.2 Calculation of Numbers of Plaques in the Cunningham Assay for Antibody secreting cells. (see also section 2.3).

The Cunningham chambers were manufactured from microscope slides (Chance Popper) which had been thoroughly cleaned with 1% concentrated HCl in absolute alcohol followed by rinsing in tap water and finally in acetone. Two slides were firmly stuck together with 3 strips of double sided adhesive tape (J.Kilby and Son No.4000DC) as illustrated in figure 2.20. Thus each pair of slides forms two chambers. The volume of each chamber was calculated by weighing a sample of chambers individually before and after filling with distilled water. The density of water was assumed to be 1g/ml, thus the volume of each chamber was calcuable from the weight gain observed on filling the chamber. It was found that the mean chamber volume was 0.0812ml with a varience less than 0.01%. The plaques were routinely scored from enlarged photographs of the chambers made by placing the chambers in an adapted negative carrier of a quarter plate enlarger. A piece of perspex, etched with a 1cm grid was placed over the photographic paper during printing to facilitate counting. Ten squares were counted on each photograph of a chamber and the results were then calculated as follows:

A) Direct Plaques.

Total Volume of mixture from which chambers are filled = 0.65ml.

Volume of spleen cells in this mixture = 0.5ml.

Volume of each chamber = 0.081ml

No. of squares on photograph covering each chamber = Y

No. of squares in which plaques counted = X

$$\text{Spleen volume in X squares} = \frac{X}{Y} \times 0.081 \times \frac{0.5}{0.65} = Z$$

Since 10 squares per chamber were counted and duplicate slides prepared

$$Z = \frac{40}{27.4} \times 0.081 \times \frac{0.5}{0.65}$$

$$= \underline{0.091}\text{ml.}$$

and Number of Plaques/ml $= \frac{\text{No. Plaques in X}}{Z}$

or No. Plaques/10^6 cells $= \frac{\text{No. Plaques in X}}{Z \times \text{No. of Millions cells/ml}}$

B) Indirect Plaques.

To visualize these plaques it is first necessary to convert the IgG secreted into a form which will fix complement and therefore result in lysis of the erythrocyte target cell. This was achieved by adding goat anti rat IgG which binds together two IgG molecules thereby creating a complement fixation site (see section 1.6). However, anti rat IgG also inhibits the development of direct plaques. It was therefore necessary to titrate the antiserum before use to determine a), the concentration at which maximum development of indirect plaques was achieved and b), the inhibition of direct plaques which occurred at this concentration.

(1) Titration of antiserum to determine concentration for maximum development of IgG PFC. (Wortis, Taylor & Dresser, 1968).

Rats were immunized 10 days prior to use with 1ml of 10% SRBC per 200g rat. The plaque assay was performed (see section 2.3 and fig.2.18) using 25 µl of Goat anti rat IgG (Miles) at one of a series of dilutions. The results show (fig.4.4.) that at high concentration the development of plaques is severely inhibited. There is a region between $1/2^3$ and $1/2^7$ where development is greater than 70% with a peak development at around $1/2^4$. At lower doses of antiserum progressively less than maximum development is achieved.

(2) The Inhibition of Direct Plaques.

The inhibition of direct plaque formation over a similar range of concentrations was also investigated. Rats were immunized 2 days prior to use with SRBC. There are very few PFC in the spleen at this time (see fig.2.23) but those which are present will almost certainly

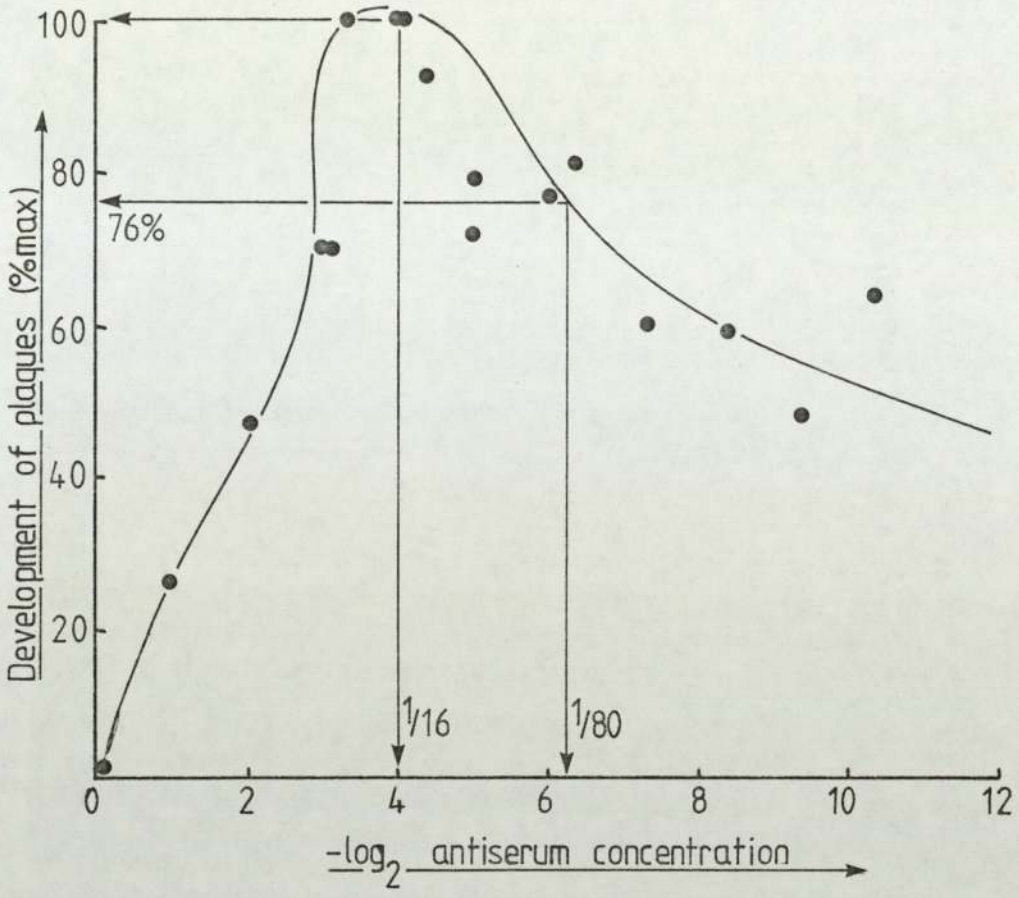


Figure 4.4. The development of IgG secreting plaques by anti IgG. The optimum development was fount to occur at $1/2^4$ dilution. At higher doses a sharp fall off in the extent of the development was seen. At dilutions greater than $1/2^4$ there was a gradual decrease in development of indirect plaques. Values are means of duplicate samples and the curve was fitted by eye. At a dilution of 1/80 the development was about 78% maximal.

be secreting IgM since this response precedes that of IgG slightly (see fig.2.19 and 1.20). Duplicates were set up with and without antiserum. From the counts obtained the inhibition constant (KI) can be calculated as follows:

$$KI = \frac{\text{Number of Plaques observed in the presence of Antiserum}}{\text{Number of Plaques in untreated Sample}}$$

The results show (fig.4.5)that this antiserum was inhibitory over a wide range. It was desirable to choose a dilution which allowed the maximum development of indirect plaques with an acceptable level of inhibition of direct plaques. The maximum development of indirect plaques occurred when using a dilution of $1/2^4$ i.e. 1/16. At this concentration however, KI for direct plaques was unacceptably low (approximately 0.45) It was decided to use a lower dilution, as a compromise, to obtain a higher value of KI with a small loss of developing capacity. An initial dilution of 1/80 was chosen which provided a KI of 0.703 and gave 76% of maximal development of indirect plaques. An initial dilution of 1/80 corresponded to a final dilution factor of 1/2080 and an immunoglobulin concentration of 2.88µg/ml.

(3) Calculation of Numbers of Indirect Plaques.

The numbers of indirect plaques cannot be obtained simply by subtracting the number of direct PFC from the total number obtained when antiserum is added because of the inhibition of the direct PFC. The following manipulation was made:-

Total PFC - (Direct PFC x KI) = Indirect PFC.

All results were expressed as PFC/million viable cells.

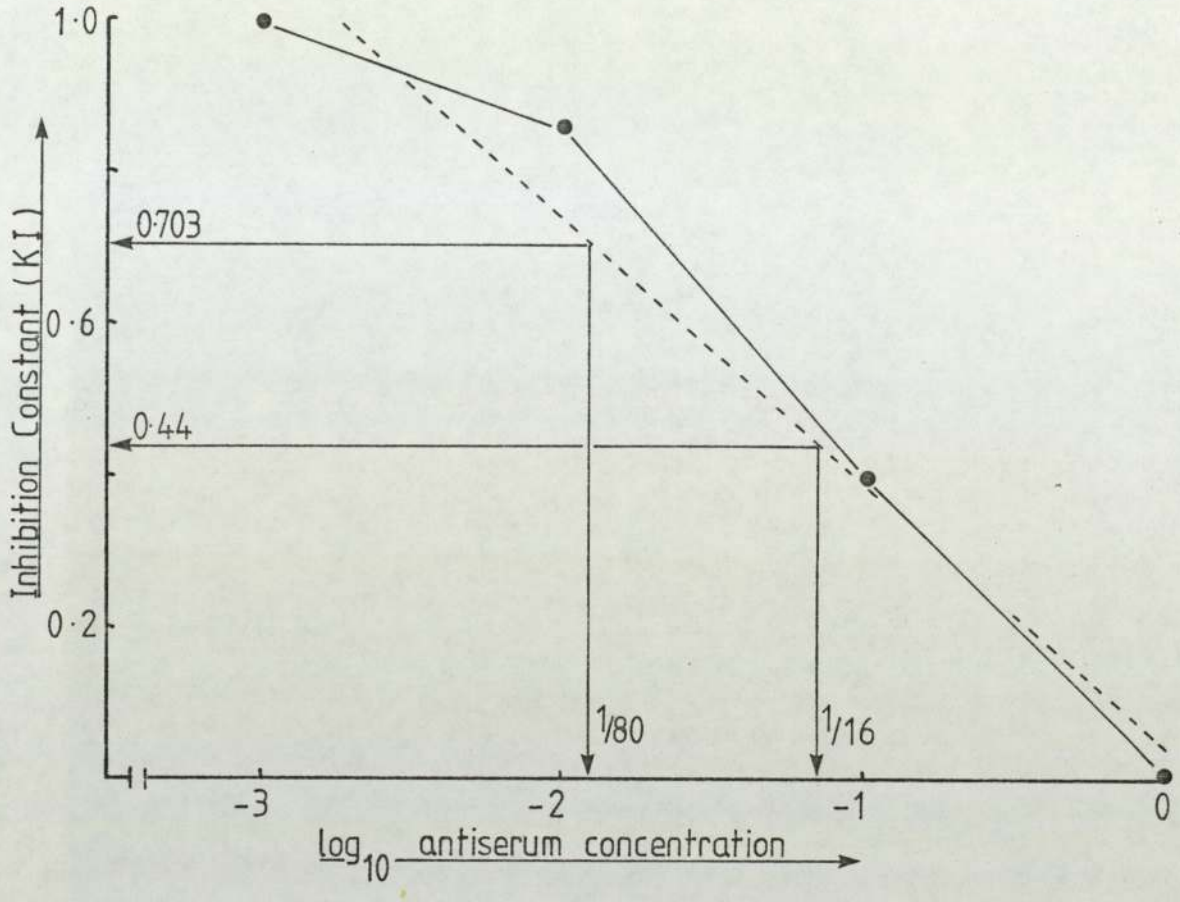


Figure 4.5. The inhibition of direct plaques by goat anti rat IgG. The inhibition constant (KI) tends towards 1.0 with less inhibition. When 25 µl of undiluted antiserum was added to the splenic lymphocyte suspension, it resulted in total inhibition. In contrast, if first diluted 1/1000 with medium KI = 1.0 i.e. no inhibition occurred. The points represent means of six replicates and the dotted line was fitted by linear regression (r[reg]= 0.98; slope = 0.238). At a dilution of 1/80 KI = 0.703.

Section 5.

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