ADVANCES IN

IMMUNOLOGY

VOLUME 39

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Immunological Regulation of Hematopoietic/Lymphoid Stem Cell Differentiation by Interleukin 3

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I. Introduction

During the past several years it has been demonstrated that the immune system regulates a number of facets of hematopoietic and lymphoid growth and differentiation. These effects are primarily mediated by a number of growth factors, termed lymphokines, which are produced by antigen-activated T cells. One of the first T cell-derived lymphokines to be extensively characterized was interleukin 2 (IL-2). This growth factor was detected by its ability to induce the proliferation of T cells *in vitro* (Morgan *et al.*, 1976). It was subsequently demonstrated that IL-2 could expand antigen-specific cytotoxic T cells *in vitro* (Gillis and Smith, 1977). From these studies, a number of cell lines were derived which continued to require IL-2 for growth (Gillis *et al.*, 1978). These lines provided a rapid and sensitive assay for IL-2 which facilitated the biochemical characterization of IL-2 and the cloning of the gene. A variety of studies have demonstrated that IL-2 regulates the differentiation and/or the proliferation of mature T cells (for review see Smith, 1980). More recently evidence has been obtained to suggest that IL-2 may also affect B cells (Mingari *et al.*, 1984).

The role of T cell factors in the regulation of B cell activity was initially shown in studies by Dutton *et al.* (1971) and by Schimpl and Wecker (1972). Since that time extensive studies have been directed at determining the number of factors involved and their specific functions. Recent studies have suggested that factors exist which stimulate the proliferation of activated or nonactivated B cells, induce the differentiation of B cells, or both (reviewed in Howard *et al.*, 1984). The factors involved in proliferation have been termed B cell growth factors (BCGFs) or B cell stimulatory factors (BSFs). The factors associated with differentiation are referred to as B cell differentiation factors (BCDFs). Extensive research is currently in progress to purify these factors to homogeneity for detailed biochemical characterization.

In addition to the factors affecting T cells and B cells, lymphokines are

produced by activated T cells which induce the proliferation and differentiation of hematopoietic cells. Historically the study of factors affecting hematopoietic cells has relied on assays measuring the proliferation and/or differentiation of bone marrow cells in soft agar cultures (Pluznik and Sachs, 1966). Initially this activity was associated with a single factor termed colony-stimulating factor (CSF). More recently it has been demonstrated that a variety of CSFs exist which, in part, can be classified by the hematopoietic lineages that are induced (reviewed in Metcalf, 1984). The majority of studies with CSFs have not involved T cells and only recently has it become fully appreciated that T cells are a source of CSF activities and that the T cell CSFs may represent a unique group of growth factors, distinct from the non-T cell-derived CSFs.

Among the factors that regulate hematopoietic stem cells which have been characterized, the non-T cell factors include CSF-1, erthropoietin, and G-CSF (granulocyte-CSF). The first CSF to be extensively characterized was CSF-1, a glycoprotein of 70 kDa composed of identical subunits of 30 kDa (Stanley and Guilbert, 1981). CSF-1 induces the proliferation of cells committed to the monocyte lineage and supports their terminal differentiation. Erthropoietin is a 34 kDa glycoprotein which induces the proliferation of cells committed to the erythroid lineage and supports the terminal differentiation of erthrocytes (Miyake *et al.*, 1977). G-CSF is a 24 kDa glycoprotein which induces the proliferation of cells committed to the granulocytic lineage and supports their terminal differentiation (Nicola *et al.*, 1983). These factors are conserved and are involved in the constitutive regulation of hematopoiesis which is independent of the immune system.

In contrast to the above factors another CSF activity, termed GM-CSF (granulocyte/macrophage CSF), is produced by activated T cells. This factor supports the proliferation and terminal differentiation of cells committed to the myeloid lineage. As detailed below, this factor has been extensively characterized and represents the major CSF activity produced by activated T cells. The available data support the concept that GM-CSF constitutes one of the major immunological mediators of the terminal stages of myeloid differentiation.

In studies directed at identifying factors that are involved in early T cell differentiation, a factor was identified which induced the expression of a T cell associated enzyme 20α-hydroxysteroid dehydrogenase (Ihle *et al.*, 1981a). This factor was purified to homogeneity and termed IL-3 based on its predominantly T cell origin and its hypothesized role in early T cell differentiation (Ihle *et al.*, 1982a). Subsequent studies demonstrated that IL-3 had the activities of a variety of T cell-derived factors (Ihle *et al.*, 1983). The broad spectrum of activities supported the concept that IL-3 regulated the growth and differentiation of early hematopoietic and lymphoid progenitors.

Moreover the T cell origin of IL-3 suggested that this factor may represent a specific immunological regulator of stem cell function.

This review will focus on the T cell factors which affect hematopoietic and T-lymphoid growth and differentiation. Particular emphasis will be given to IL-3 and GM-CSF since IL-2 has been extensively reviewed in a number of excellent articles (Smith, 1980; Robb, 1984; Smith *et al.*, 1983). The biochemical properties of the factors will be considered and compared. The interrelationship of the factors in promoting the differentiation of hematopoietic/lymphoid lineages will be described. The factors affecting production of the lymphokines by T cells will be examined and considered relative to their significance in the immunological regulation of inflammatory responses. Finally the growth regulatory properties of lymphokines will be considered with particular emphasis on the effects of various oncogenes.

II. Biochemical Properties of T Cell-Derived Lymphokines

Among the growth factors produced by activated T cells the most extensively characterized lymphokines are IL-2, IL-3, and GM-CSF. Each of these proteins has been purified to homogeneity and molecular clones encoding them have been obtained. A summary of the properties of these factors is shown in Table I. For comparison the properties of the non-T cell-derived hematopoietic growth factors are also shown.

TABLE I
PROPERTIES OF FACTORS REGULATING HEMATOPOIETIC/LYMPHOID DIFFERENTIATION AND PROLIFERATION[a]

	Constitutive regulation			Immunological regulation		
	Erythropoietin	CSF-1	G-CSF	IL-2	IL-3	GM-CSF
Molecular weight						
Native	34	37–70	25	15–30	28	23
Protein	16	15	N.D.	16	15	14
Carbohydrate	+	+	+	+	+	+
Molecular weight						
Receptor	N.D.	165	180	55	N.D.	N.D.
Cellular sources	Hepatocytes	Many cells	Unknown	T cells	T cells	T cells
Lineages affected	E	M	G	T, B	G, M, T, mast, E	G, M
Chromosome						
Human	N.D.	N.D.	N.D.	4q	N.D.	N.D.
Mouse	N.D.	N.D.	N.D.	N.D.	11	11

[a] N.D., Not determined; E, erythroid; M, monocytic; G, granulocytic; T, T cell; B, B cell.

Human IL-2 was the first lymphokine to be purified to homogeneity (Meir and Gallo, 1980). The initial procedures used standard chromatography techniques and isolation of the protein from polyacrylamide gels run in sodium dodecyl sulfate (SDS). Techniques have also been described for the isolation of primate IL-2 (Henderson *et al.*, 1983) which take advantage of the hydrophobic properties of IL-2 and use absorption and elution of the protein from derivatized glass beads. Subsequent chromatography on high-performance liquid chromatography (HPLC) columns yields a homogeneous protein. This approach has been adapted for the purification of murine IL-2 (Henderson *et al.*, 1983; Keller, unpublished data). One of the most efficient purification techniques employs IL-2 affinity columns using monoclonal antibodies against IL-2 (Robb *et al.*, 1983).

The structure of IL-2 has been primarily deduced from the sequence of cDNA clones. Taniguchi *et al.* (1983) first reported the isolation of a IL-2 cDNA clone which was detected by the ability to hybridize mRNAs encoding IL-2 activity. Murine IL-2 has been cloned from a helper T cell cDNA expression library (Yokota *et al.*, 1985) and from a helper T cell cDNA library by using the human cDNA clones as probes (Kashima *et al.*, 1985). Human IL-2 is a single polypeptide chain of 153 amino acids of which 20 amino acids encode a hydrophobic leader sequence which is removed during processing. Murine IL-2 has a comparable structure and is a polypeptide of 169 amino acids which similarly contains a 20 amino acid leader sequence. Within the murine sequence there exists a 14 amino acid sequence for which there is no equivalent region in the human IL-2 sequence. This region contains 12 repetitions of the sequence CAG which would code for a stretch of 12 glutamines. The origin of this unique region is not known. Among the conserved sequences there exists approximately a 70% homology in the nucleotide sequences. Among the amino acid positions which are conserved in both genes 95 are identical whereas 56 positions have different amino acid residues.

Murine IL-2 has an apparent molecular weight of 30,000 during purification indicating that the native protein is a dimer (Paetkau *et al.*, 1984). In contrast, human IL-2 has an apparent molecular weight of 15,000. From the sequences there are no potential sites for N-glycosylation in either murine or human IL-2. Human IL-2, however, has been shown to contain O-glycosidic-linked *N*-acetylgalactosamine at the Thr in position 3 of the mature protein (Robb *et al.*, 1983). Murine IL-2 contains a comparable Thr residue but has not been shown to be modified. Human IL-2 contains an internal disulfide bond which has been shown to be required for activity by site-directed mutagenesis studies (Wang *et al.*, 1983). Although murine IL-2 contains an internal disulfide bond which has been shown to be required for activity by site-directed mutagenesis studies (Wang *et al.*, 1983). Although

murine IL-2 contains the same Cys residues it is not known whether disulfides are required for activity.

In humans there exists a single gene encoding IL-2 which has been localized to chromosome 4q (Seigal *et al.*, 1983). The gene is interrupted by three introns (Fujita *et al.*, 1984). In the 5′ flanking sequences there is an interesting region which shows sequence homology to the promotor region of the human γ-interferon gene. In addition, the second intron contains a region with similarity to viral enhancer elements. These sequences may play a role in the regulation of expression of the gene in T cells. The structure of the murine genomic IL-2 gene has not been described and the chromosomal location is currently not known.

IL-3 was initially identified by its ability to induce the enzyme 20αSDH in cultures of splenic lymphocytes from athymic mice (Ihle *et al.*, 1981a). Using this assay procedures were developed for the purification of IL-3 to homogeneity (Ihle *et al.*, 1982a). Standard chromatographic approaches were used and final purification was achieved using C_{18} hydrophobic columns in HPLC. The purified protein had an apparent molecular weight of 28,000 and contained approximately 12% glucosamine suggesting the presence of substantial carbohydrate (Ihle *et al.*, 1984). The specific activity of IL-3 is approximately 0.05–0.2 ng/ml for 50% of maximal activity which corresponds to approximately 10^{-12} *M*. In addition to the major 28 kDa species additional, larger molecular weight forms of IL-3 have been purified which have identical properties. Protein sequence analysis has demonstrated that these forms are due to differences in the extent of glycosylation.

The isolation of cDNA clones for IL-3 has been reported by two groups. In the inital report a cDNA clone was obtained from a cDNA library prepared from the WEHI-3 cell line by its ability to hybrid select RNA which produced a factor which could support the proliferation of an IL-3-dependent cell line (Fung *et al.*, 1984). In separate studies a cDNA clone was obtained which encoded a mast cell growth factor from a cDNA library from a concanavalin A-activated T cell line (Yokota *et al.*, 1984). The sequences of both cDNA clones were identical with the exception of one base at position 463 which is due to an allelic variation between BALB/c and C57BL/6 mice. In addition, both sequences contained a predicated protein sequence which was identical to the N-terminal sequence of purified IL-3. Based on the sequence identity, the biological activity, and the existence of a single gene by hybridization of genomic DNA with cDNA probes, it was concluded that the clones encoded the gene for IL-3.

The primary structure of IL-3, as deduced from the cDNA clones, is shown in Fig. 1. The sequence indicates the presence of a hydrophobic leader sequence. From the protein sequence analysis of purified IL-3, the NH_2-terminal of the mature protein corresponds to either Asp in position 33

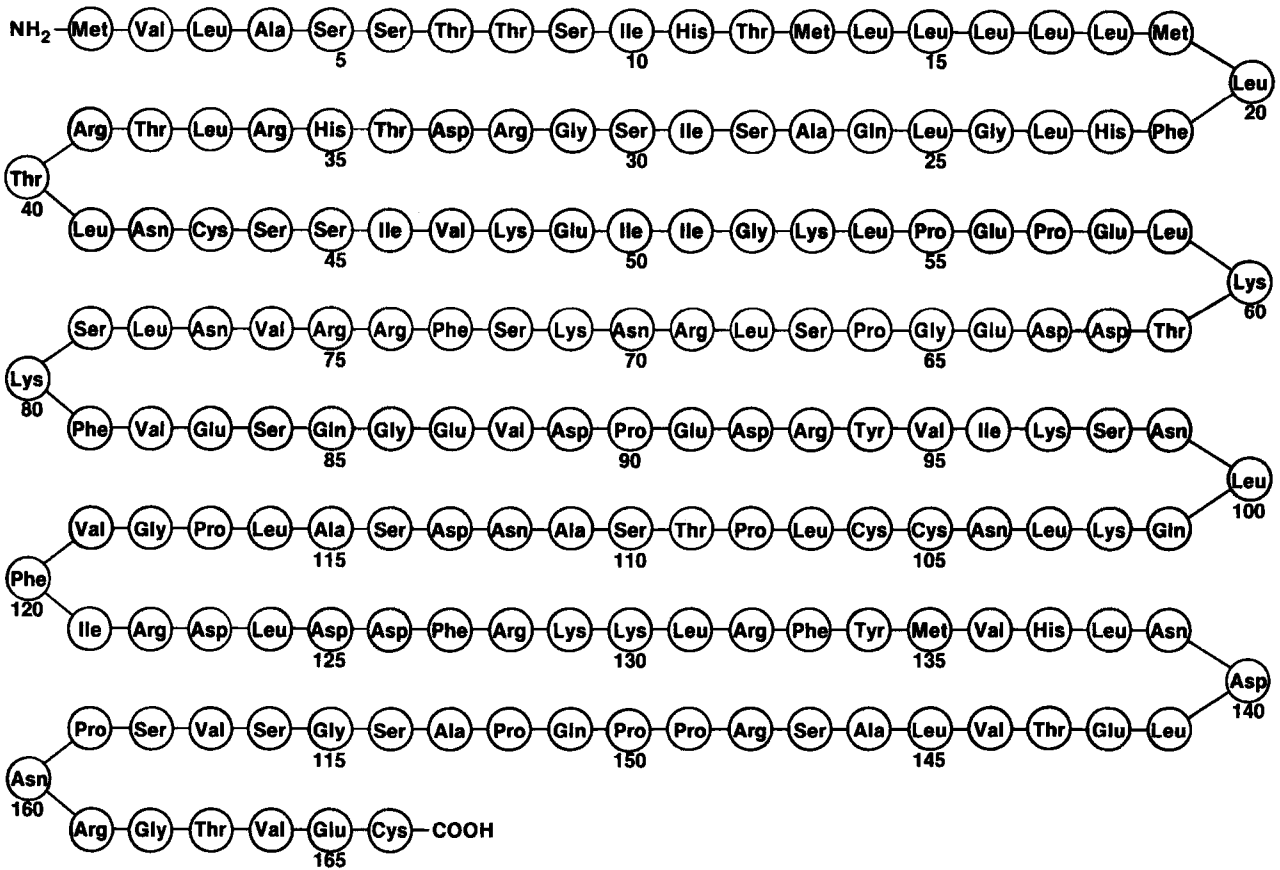

FIG. 1. Amino acid sequence of interleukin 3. The primary amino acid sequence of IL-3 shown was deduced from the published sequence (Yokota *et al.*, 1984) of a cDNA clone encoding IL-3.

or Ala in position 27. In the initial studies (Ihle *et al.*, 1983a) the amino terminal sequence was shown to start at the Asp in position 33. In a subsequent study (Clark-Lewis *et al.*, 1984), using comparable purification procedures, the amino terminal sequence began at the Ala in position 27. It is not known why differences have been observed in the amino terminal or whether these differences have biological significance. From the cDNA sequence potential N-glycosylation sites exist at residues 42, 70, 77, and 112. From the protein sequencing data no Asn residue was detectable at residue 42 which strongly suggests that in the mature protein this site is glycosylated. Whether the other sites are substituted is not known, but since IL-3 contains approximately 35–40% carbohydrate it is likely that additional residues are substituted. On the basis of the cDNA sequence the mature protein would have a molecular weight of 15,000. The difference from the apparent molecular weight of 28,000 is due to the presence of carbohydrate.

Whether the carbohydrate is required for the biological activity of IL-3 is not known. We have attempted to assess this by using trifluoromethanesulfonate to remove the carbohydrate (unpublished data). Under relatively mild conditions which did not remove the carbohydrate as determined by mobility in SDS–PAGE, the biological activity was lost. Therefore this approach could not be used to address the question. It should be noted, however, that Bazill *et al.* (1983) concluded that the carbohydrate was necessary based on the loss of activity in trifluoromethanesulfonate. The correlation between the removal of carbohydrate and the loss of biological activity, however, was not examined in the studies.

Murine IL-3 is coded for by a single gene which is detectable in Southern blot analysis of *Eco*RI-restricted DNA as a single fragment of 8.5 or 10.8 kb depending on the mouse strain (Miyatake *et al.*, 1985; Campbell *et al.*, 1985; Ihle *et al.*, 1986). The IL-3 gene is composed of four introns and five exons as illustrated in Fig. 2. A TATA like sequence is preceded by a G+C-rich region in the 5′ flanking region. G+C-rich regions have been shown to be important for the efficient transcription initiation in SV40 (Fromm and Berg, 1982) and for the herpes thymidine kinase gene (McKnight and Kingsbury, 1982). Interestingly there are nine repeats of a closely related 14 bp sequence in the second intron of the gene. These repeated sequences share homolog with a 20 bp repeated sequence in the human genome which has been postulated to have enhancer activity. Whether these sequences have significance to the expression of the IL-3 gene is unknown.

The murine IL-3 gene has been mapped to chromosome 11 using a series of mouse × hamster somatic cell hybrids (Ihle *et al.*, 1986). In addition, the restriction enzyme polymorphisms seen with various strains using *Eco*RI have been used to map the gene in backcrossed mice. In these studies IL-3 was shown to be linked (20± 7% recombination) to the c-*erb*B gene using a restriction enzyme polymorphism in the c-*erb* gene. Finally, IL-3 was found

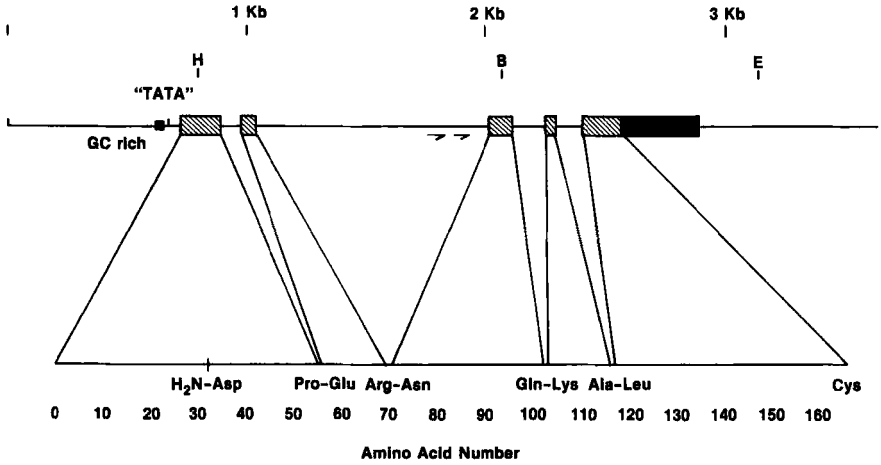

FIG. 2. Genomic organization of the interleukin 3 gene. The structure of the IL-3 gene was that described by Miyatake *et al.* (1985). Exons containing coding regions are indicated by hatched boxes. The solid boxed area is the 3′ noncoding region. The positions of the exons relative to the native protein are indicated by the amino acids and amino acid numbers shown on the bottom. Restriction enzyme sites for *Hin*dIII (H), *Bam*HI (B), and *Eco*RI (E) are indicated.

to be linked to the *nu* locus by examining strains in which the *nu* locus had been introduced through successive inbreding. The location on chromosome 11, however, has not been definitely established. Interestingly, none of the mutations which affect hematopoiesis including the *steel* locus, the *W* locus, or the gene for Hertwig's anemia maps to chromosome 11. However, in addition to the *nu* locus and the c-*erb*B locus, chromosome 11 contains the *Hba* locus which codes for the hemoglobin α complex, and, as noted below, the locus for GM-CSF.

To date the human homolog to the murine IL-3 gene has not been described. In the biological assays that have been used to detect murine IL-3, factors from potential sources of human IL-3 have not reproducibly yielded activity that could be purified and characterized. Conversely, attempts have been made to demonstrate an effect of murine IL-3 on human cells without success. Although reports of cross-reactivity exist this has not been a consistent observation. Alternatively cDNA probes for murine IL-3 have been used to examine human genomic DNA for cross-reactive sequences under relatively low stringencies without success to date. Therefore, there currently exists no evidence that a human counterpart to the murine IL-3 exists. If, as is likely, however, a homolog is present, it can be concluded that the gene is not highly conserved and will show either a comparable degree

homology or less than that observed with IL-2 or GM-CSF between the murine and human genes.

The initial studies of the biochemical and biological properties of GM-CSF were with factor purified from conditioned media from lungs of mice treated with endotoxin (Burgess *et al.*, 1977). For historical reasons mouse lung conditioned media had been characterized as a source of a variety of hematopoietic growth factors. The assays used for purification relied on the ability to induce colonies of granulocytes and macrophages in soft agar cultures in a 7 day assay. With this assay the protein was purified to homogeneity using standard purification procedures. Purified GM-CSF has a molecular weight of 23,000 and is half maximally active at concentrations of 10^{-12} *M* comparable to other growth factors. The presence of carbohydrate was shown by direct analysis of the purified protein.

The cloning of GM-CSF was accomplished through the use of oligonucleotide probes predicted from the amino-terminal sequence of the protein (Gough *et al.*, 1984). These probes were used to screen a cDNA library prepared from poly(A) RNA isolated from lung tissues of endotoxin-treated mice. The largest clone obtained lacked an initiation codon suggesting that the clone did not contain the 5′ region of the RNA. The primary structure of the protein deduced from the cDNA sequence has a molecular weight of 13,500 which is in good agreement with the apparent molecular weight of 16,800 for deglycosylated GM-CSF. Two potential N-glycosylation sites occur within the predicted amino acid sequence. There are no apparent sequence homologies between murine GM-CSF and murine IL-3 or IL-2.

By Southern blot analysis, GM-CSF is coded for by a single gene in mice. Using a series of mouse × hamster hybrids initial data suggested that the gene may be on chromosome 11 (Gough *et al.*, 1984). In additional studies (Ihle and Arai, unpublished data) using an independently derived panel of somatic cell hybrids, we have also mapped GM-CSF to chromosome 11. This is of particular interest because IL-3, as indicated above, has been mapped to chromosome 11 suggesting the possibility that some of T cell-derived growth factors may constitute a family of genetically linked genes. In this regard it should be noted that the gene for murine IL-2 has not yet been genetically mapped.

More recently the human gene equivalent to murine GM-CSF has been purified to homogeneity and cDNA clones have been obtained (Wong *et al.*, 1985). The cloning made use of an expression vector and detection of the production of biological activity in COS-1 monkey cells. The library was derived from membrane-bound mRNA from a lectin-stimulated human T cell line derived from a hairy cell leukemia. The structure of GM-CSF, deduced from the cDNA clone, consists of a protein of 144 amino acids. A hydrophobic leader sequence of 17 amino acids is removed in the mature

protein. The sequence contains a single potential site for N-glycosylation. Purified human GM-CSF has a heterogeneity in size and in charge which is consistent with the presence of carbohydrate.

The sequence of human GM-CSF shows homology with the murine homolog. There is approximately a 60% homology in the protein sequences and approximately a 70% homology in the nucleotide sequences. Interestingly the positioning of the four cysteine residues has been preserved in the two proteins suggesting some importance for the location of disulfide bonds comparable to IL-2. There is no sequence homology between human GM-CSF and human or murine IL-2 or with murine IL-3. By Southern blot analysis, human GM-CSF is coded for by a single gene which has not been genetically mapped.

A comparison of the structure of the cDNA clones for both human and mouse-derived T cell factors shows considerable similarity. In particular each is approximately 1–1.2 kb and contain a relatively short 5′ untranslated region. All the factors have polypeptide chains of comparable size and the molecular weight variations which occur among the mature proteins are due to dimerization or carbohydrate substitution. In all the cDNA clones there is a relatively large 3′ untranslated region of 270–320 bases. In spite of the general structural homology, however, there is no indication from the sequences that the various RNAs share regions of sequence homology. Nor is it clear yet that the genomic organization will have shared features although only the murine IL-3 and human IL-2 genomic structures have been sequenced. These aspects will be of considerable interest as studies are directed to the regulation of production of these lymphokines.

III. Cellular Sources of Interleukin 3

The cellular origins of IL-3 as well as GM-CSF are similar to IL-2. First IL-3 and GM-CSF are predominantly produced by T cells, although, as noted below, some interesting exceptions exist. Second, the available evidence suggests that the antigen or mitogen-induced production of these lymphokines occurs coordinately. Third, factors which affect the production of IL-2 by helper T cells also affect the production of IL-3 and GM-CSF. Taken together, the concept emerges that a unique subpopulation of antigen-specific T cells exists which function to coordinately produce a group of growth factors which includes IL-3, GM-CSF, and IL-2.

Like IL-2, IL-3 and GM-CSF are predominantly products of antigen or mitogen-activated T cells. In the initial studies (Ihle *et al.*, 1981a) it was shown that T cell mitogens induced the production of IL-3 whereas production was not observed with B cell mitogens in cultures of normal splenic lymphocytes. The predominantly T cell origin was further indicated by the

lack of detectable production of IL-3 in cultures of mitogen stimulated splenic lymphocytes from young athymic mice. In studies of the immune responses to Moloney leukemia virus (MoLV), IL-3 was shown to be produced in specific response to viral antigens in a dose dependent manner by Thy-1$^+$, Lyt-1$^+$,2$^-$ lymphocytes (Ihle *et al.*, 1981b). In this particular system antigen-presenting cells were not found to be required for the production of either IL-2 or IL-3. In systems in which there has been demonstrated to exist an Ia restriction for helper T cell function, the production of IL-3 is dependent on the presence of appropriate antigen-presenting cells (Prystowsky *et al.*, 1982).

In contrast to IL-3, GM-CSF has not been considered to be predominantly a T cell product. In the original studies conditioned media from lungs of mice treated with endotoxin were shown to contain large quantities of activity which induced colonies of granulocytes and macrophages (Sheridan and Metcalfe, 1973). It was subsequently demonstrated that conditioned media from several tissues contained a GM-CSF-like activity with similar biochemical properties (Nicola *et al.*, 1979). The cellular sources of this activity in the tissues, however, were not identified. The ability of mitogen-activated T cells to produce a comparable activity was demonstrated by Burgess *et al.*, (1980). Using a variety of cell lines, no non-T cells lines have been found to produce GM-CSF whereas a variety of T cell lines can be induced to produce large quantities of GM-CSF. Therefore the question remains as to whether the same GM-CSF produced by activated T cells is produced by other cell types.

In order to better understand the regulation of immune responses it becomes important to know whether there exists a single class of functional T cells which produce lymphokines, whether these cells produce the same spectrum of lymphokines, whether the relative levels of the various factors can be altered, and finally, whether there exist genetic factors which can influence the relative levels of expression of the genes for various lymphokines. These questions have been approached by (1) examining normal, resident T cell populations using limiting dilution analysis, (2) examining T cell clones that have been expanded *in vitro* for limited times, or (3) examining long-term T cell lines.

Using a limiting dilution analysis Miller and Stutman (1983) initially examined the characteristics and frequencies of IL-2-producing cells to alloantigens. The results demonstrated that in the system used, there was a linear relationship between the dilution of cells and the frequency of IL-2-producing cells suggesting that a single cell could be detected with the system. Using this assay they demonstrated that there was approximately a 10-fold higher frequency of IL-2 producer cells in the Lyt-2$^-$ population relative to the Lyt-2$^+$ population of T cells. In subsequent studies (Miller and Stut-

man, 1983) it was found that the frequency of cells producing IL-2 in response to Con A was 17-fold higher in the Lyt-2$^-$ population. When analyzed for IL-3 production, it was found that all cells thQt produced IL-3 also produced IL-2. Furthermore, the amount of IL-2 produced was strongly correlated with the amount of IL-3 produced. These results therefore suggested that a single class of T cells exists which coordinately produces both lymphokines.

The results obtained with clones of T cells expanded for limited times *in vitro* are more complex. Kelso and Metcalf (1985) examined 55 alloreactive T cell clones for the production of IL-3, IL-2, and GM-CSF. Among these, 35% of the Lyt-2$^+$ clones produced IL-2 or IL-3 and 46% produced GM-CSF or IL-3 (IL-3 could not be discriminated from GM-CSF in the assays used). Among the L3T4$^+$ clones, all produced GM-CSF or IL-3, whereas 57% produced IL-3 and 57% produced IL-2. When 26 clones were examined which produced either IL-2 or IL-3, 27% only produced IL-2 while 12% only produced IL-3. In addition, there was considerable variation in the relative levels of IL-3 and IL-2 activity. Therefore, unlike the conclusions from limiting dilution analysis, there was little correlation between phenotypes of T cells producing lymphokines or their coordinant production. The basis for the differences is not known but in the latter studies the cells were expanded *in vitro* with added IL-2, in the presence of irradiated, non-T cell-depleted, spleen cells which may contribute to the differences.

Using a series of T cell clones, derived by stimulation of T cells with Con A, Guerne *et al.* (1984) examined the correlation between IL-2, IL-3, and interferon production. Among clones of Lyt-2$^-$ cells there was a good correlation between IL-2 and IL-3 production in that all produced both lymphokines and 10/13 also produced interferon. In examining Lyt-2$^+$ clones, there was either no production of lymphokines or only low levels and no general correlation for the production of individual lymphokines.

Using long-term T cell lines, maintained in the absence of irradiated filler cells, Nabel *et al.* (1981a) initially demonstrated that specific subpopulations of T cells were capable of producing a variety of lymphokines. The clone characterized (Ly1$^+$2$^-$/9) has subsequently been shown to specifically produce IL-3, GM-CSF, as well as IL-2 following mitogenic stimulation. Similarly the L2 T cell clone initially isolated by Glasebrook and Fitch (1980) was subsequently shown to produce GM-CSF in addition to IL-2 (Ely *et al.*, 1981). In this study it was demonstrated that a variant could be obtained which had lost the ability to produce detectable levels of IL-2. It has also been demonstrated that the L2 line produces IL-3 (Prystowsky *et al.*, 1982).

The spectrum of lymphokines produced by T cell hybridoma cell lines has been examined (Harwell *et al.*, 1980; Burgess *et al.*, 1981; Schrader *et al.*,

1982; Zlotnik *et al.*, 1983). In these cases, as above, the majority of the lines have been found to produce a variety of lymphokines including IL-2, IL-3, interferon and factors affecting B cell growth or differentiation. Where examined, the relative ratios of the various factors appear to be comparable. Taken together, therefore, the concept emerges that a single class of T cells exists which functionally is characterized by the ability to produce a "family" of lymphokines in response to antigen activation. Moreover, the relative levels of production may be constant suggesting coordinate regulation.

Several lymphoma cell lines have been shown to produce IL-2 following stimulation with mitogens or phorbol esters. These include the EL-4 lymphoma which was initially shown to produce IL-2 following stimulation with mitogens (Shimizu *et al.*, 1980) and following stimulation with phorbol esters (Farrar *et al.*, 1980). This cell line also produces IL-3 and GM-CSF following stimulation although the levels of IL-3 are low relative to other normal mitogen-stimulated cells (Keller and Ihle, unpublished data). In addition the LBRM-33 lymphoma cell line was shown to be inducible for the production of IL-2 (Gillis *et al.*, 1980) and was shown to coordinately synthesize a colony-stimulating factor activity (Watson, 1983). In more recent studies (Prestidge *et al.*, 1984), it was shown that the LBRM-33 cells produced three chromatographically distinguishable CSF activities. One appeared to have the properties of IL-3 which another had the properties expected of GM-CSF. Interestingly a variant of the LBRM-33 line was described which constitutively produces IL-2. The basis for this has not been established and the line has not been further characterized.

With the exception of T cell lines, few cell lines have been shown to produce IL-3. As described in detail below the most noticable exception has been the constitutive production of IL-3 by the WEHI-3 cell line. In recent studies (Luger *et al.*, 1985) it was reported that normal epidermal cells or a mouse keratinocyte cell line produced an IL-3-like activity. Whether this factor is IL-3 cannot be definitively assessed from the data presented. In particular, the factor was not purified and the levels of activity were extremely low relative to the activity observed with WEHI-3 conditioned media. It is unlikely that the factor is IL-3, however, since an antiserum, which inhibits IL-3 activity completely (Bowlin *et al.*, 1984), only partially inhibited the activity and required higher immunoglobulin concentrations than are required for IL-3 inhibition.

IV. Regulation of the Production of Interleukin 3

The production of lymphokines by mitogen or antigen-activated T cells requires RNA synthesis and appears to involve the induction of transcription of the genes. This has been suggested by Northern blot analysis of RNAs

from noninduced and induced T cells using cDNA probes for IL-2 (Taniguchi *et al.*, 1983; Yokota *et al.*, 1985; Kronke *et al.*, 1984; Kashima *et al.*, 1985), IL-3 (Yokota *et al.*, 1984), and for GM-CSF (Gough *et al.*, 1984; Wong *et al.*, 1985). Induction leads to the rapid accumulation of RNA. In the case of mitogen-induced production of IL-3, mRNA accumulation is detectable within 2 hours and increases for 24–36 hours. Where examined, the kinetics of accumulation of RNA for IL-2 and for GM-CSF have been shown to be comparable. Using inhibitors of DNA synthesis such as mitomycin C it has been demonstrated that the mitogen-induced production of IL-3, GM-CSF, or IL-2 is not inhibited. Although the results suggest that mitogen or antigens act to directly induce the transcription of the RNAs for IL-2, IL-3, or GM-CSF it is conceivable that mechanisms involving mRNA stabilization are involved. This aspect can, in part, be addressed using nuclear runoff assays which measure the level of transcription of specific genes (Groudine *et al.*, 1981).

Glucocorticoids are known to be immunosuppressive and antiinflammatory. One possible mechanism for this has been suggested by the effects of glucocorticoids on the production of lymphokines by helper T cells. Gillis *et al.* (1979) initially suggested that glucocorticoids could inhibit the production of IL-2 by activated helper T cells. In more recent studies Arya *et al.* (1984) have shown that dexamethasone inhibits the accumulation of IL-2 mRNA in mitogen-stimulated peripheral blood lymphocytes. In studies which used the Lyt-1^+2^-/9 clone of helper T cells it was demonstrated that dexamethasone inhibited the appearance of IL-3 activity and the accumulation of IL-3 mRNA in the cells (Culpeper and Lee, 1985). In addition, a similar effect was observed in the accumulation of mRNA for GM-CSF. In contrast, dexamethasone had no effect on the accumulation of Thy-1 mRNA demonstrating that the effect was specific for mitogen-inducible genes. The observation that dexamethasone can specifically suppress the induction of helper T cell-derived lymphokines will have significant implications in understanding of the effects of glucocorticoids *in vivo*.

The production of IL-3 by T cells, like the production of IL-2 (Hess *et al.*, 1982), is blocked by the fungal product cyclosporin A (Orosz *et al.*, 1983; Keller and Ihle, unpublished data). This cyclic peptide was initially isolated by its immunosuppressive effects in animal models (Borel *et al.*, 1977). With antigen or mitogen activated T cells, concentrations of approximately 10^{-9} *M* will cause half maximal inhibition of IL-3 production. These concentrations are comparable to those required for the inhibition of IL-2 production as well as GM-CSF production (Keller and Ihle, unpublished data). The mechanism of action is not known although it has been shown that the drug functions to block the appearance of IL-2 mRNA by Northern analysis (Kronke *et al.*, 1984). In addition, cyclosporin A does not inhibit the production of IL-3 by

WEHI-3 cells (Keller and Ihle, unpublished data) which constitutively produces IL-3 as described below, indicating that cyclosporin A does not inhibit the synthesis and release of lymphokines. Taken together therefore the data suggest that cyclosporin A specifically interrupts the antigen or mitogen-induced signal which is required for the induction of transcription. The comparable effects on IL-3, IL-2, and GM-CSF suggest that the inhibition is of a general regulatory component of this gene set perhaps at the level of the T cell receptor.

One of the useful sources of IL-3 has been the WEHI-3 cell line (Lee *et al.*, 1982). The cells have been characterized as myelomonocytic by morphology. Unlike other known myelomonocytic cell lines, however, the WEHI-3 cells produce high levels of IL-3. Unlike the T cell lines which have been characterized, the WEHI-3 cells do not require an inducing stimulus but rather constitutively produce IL-3. For this reason WEHI-3 cells have been extremely valuable for the large scale production of conditioned media for the purification of IL-3. Also, unlike T cell lines, the WEHI-3 cells do not produce other known lymphokines including IL-2 or GM-CSF. It should be noted, however, that a recent report indicated that the WEHI-3 cells produce factor(s) which affect B cell growth and differentiation (Booth *et al.*, 1983). Whether these factors are comparable to T cell factors for B cell growth is not known. Also, as noted above, the WEHI-3 differs from T cells in that cyclosporin A does not inhibit the production of IL-3.

The possible reasons for the unusual ability of the WEHI-3 cells to produce IL-3 has come from Southern blot analysis of restriction enzyme-treated DNA (Fig. 3, Ihle and Gilbert, unpublished data). In normal BALB/c DNA, the IL-3 gene is contained on a single *Eco*RI restriction fragment of 8.5 kb (Miyatake *et al.*, 1985). The WEHI-3 DNA contains, in addition to the normal fragment, a rearranged allele in which the IL-3 gene is found on an *Eco*RI fragment of 4 kb. In restriction enzyme mapping of the rearranged allele, the change is due to rearrangement of a region which is approximately 300 bases 5′ to the promoter region of the gene. The precise characteristics of the rearrangement have been studied by molecular cloning (Ymer *et al.*, 1985). The rearranged allele was found to contain an insertion of an endogenous intracisternal A particle genome 215 bases 5′ of the putative IL-3 TATA box. The transcriptional orientation of the inserted genome is opposite that of the IL-3 gene. This observation, and the observation that transcription of the IL-3 gene in WEHI-3 cells initiates at the same site as in T cells, suggests that the retroviral genome functions to enhance expression from the rearranged locus. The presumed enhancer activity of the retroviral insertion was confirmed by comparing the ability of the rearranged allele with the normal allele to be expressed in COS-1 cells after transfection. In this assay only cells transfected with the rearranged allele expressed IL-3.

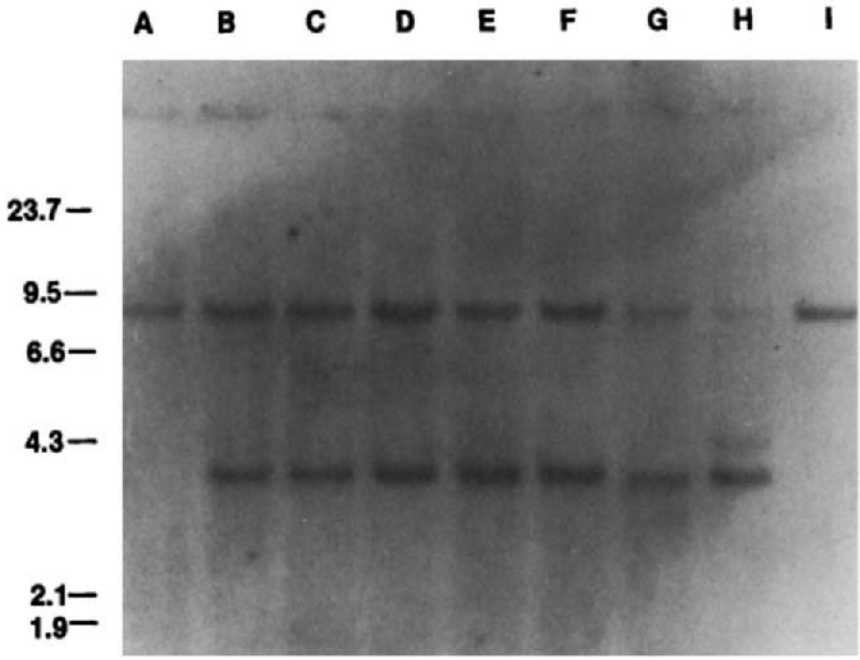

FIG. 3. Southern blot analysis of *Eco*RI-restricted DNAs from various cell lines. DNA was obtained from the indicated cell lines and restricted with *Eco*RI. The DNAs were electrophoresed in 0.8% agarose gels, the DNA transferred to nitrocellulose, and the filters hybridized with an IL-3 cDNA probe. The cell lines examined included A, EL-4; B, SNVC43; C, SA47C1; D, 3BA4; E, 3BA3; F, 3BA2; G, A4A; H, WEHI-3; I, DNA from BALB/c splenocytes. The cell lines B–G were derived in previously described studies (Hapel *et al.*, 1981).

The origin of the WEHI-3 cell line suggests the possible conditions which could have selected for a cellular variant capable of producing IL-3. In particular, the WEHI-3 line was established in tissue culture from a animal passaged tumor cell line derived from a chloroleukemia (Warner *et al.*, 1969). Recently it has been shown that a number of myeloid leukemias and, in particular, a chloroleukemia require IL-3 for growth *in vitro* (Holmes *et al.*, 1985). Interestingly the IL-3-dependent myeloid cell lines like the WEHI-3 cells express high levels of Thy-1. This expression is characteristic of stem cells at a point in differentiation when cells become committed to the myeloid lineage (discussed below). Therefore, it is likely that the WEHI-3 tumor was initially IL-3 dependent and that either *in vitro* or *in vivo* there was selection for variants capable of producing a requisite growth factor. Presumably, however, other changes have occurred since the current

WEHI-3 cells do not have receptors for IL-3 and their growth is not inhibited by antiserum against IL-3 (Palazynski and Ihle, 1984).

It is not currently known whether examples comparable to the WEHI-3 cell line exist for other lymphokines. In this regard, however, it would be of interest to examine the variant of the LBRM-33 cells described by Stull and Gillis (1981). In addition, Rabin *et al.* (1981) have shown that a primate T cell line (MLA144) constitutively produces IL-2. In this case it is conceivable that the cells are either altered specifically for IL-2 production or may produce IL-2 due to effects associated with the normal mechanisms by which mitogen or antigens induce transcription of the lymphokine genes. The possible involvement of rearrangements is suggested by the observation that the production of IL-2 is not inhibited by cyclosporin A comparable to the IL-3 production by the WEHI-3 cell line (D. Kaplan, personal communication).

In a series of studies dealing with clonal lines of P cells (persisting cells or mast cells), it was observed that variant cells could be obtained which grew without the addition of a factor termed P cell growth factor (Schrader and Crapper, 1983). As noted below, this factor has subsequently been found to be IL-3. The factor-independent lines were found to constitutively produce readily detectable levels of the growth factor suggesting that the emergence of factor-independent lines involved the selection for variants capable of producing their own growth factor. Whether this phenomenon is associated with rearrangements in the IL-3 gene comparable to the WEHI-3 cell line and the constitutive production of IL-3 was not examined.

It has been reported (Hapel *et al.*, 1981) that a series of Thy-1$^+$, Lyt-1$^+$,2$^-$ cell lines constitutively produce IL-3. These cell lines were isolated from cultures of normal splenic lymphocytes which contained WEHI-3 conditioned media. In subsequent studies, it has not been possible to isolate comparable cell lines (Ihle, unpublished data). By Southern blot analysis of several of the cell lines, the same *Eco*RI fragment is observed that has been observed in the WEHI-3 cell line (Fig. 3B–G). The cell lines differ, however, in that they express Lyt-1 whereas the WEHI-3 cell line is Lyt-1$^-$. In addition, none of the cell lines has a rearranged T cell receptor β gene whereas the WEHI-3 cell line does (Freganaro and Ihle, unpublished data). Consequently, the origin of the cells is problematic but may represent a contaminant from the original WEHI-3 cell line used to prepare the conditioned media.

Taken together, the data demonstrate that IL-3 is predominantly, if not exclusively, a product of activated T cells and appears to represent one of a group of coordinately regulated lymphokines which includes IL-2 and GM-CSF. This set of factors represent the functional products of a unique subpopulation of antigen-specific T cells which are induced by antigens or

mitogens. The observation that both IL-3 and GM-CSF genetically map to chromosome 11 suggests the intriguing possibility that this group of factors may be genetically linked which in turn may be important for their coordinate regulation. One of the interesting questions is whether there exist genetic differences in the regulation of production of these factors which may be important for strain variations in the types of immunological responses that are observed.

V. Multiple Biological Activities Associated with Interleukin 3

Over the past several years conditioned media from T cells has been shown to mediate a variety of activities which were ascribed to individual factors. With the purification of lymphokines to homogeneity it became obvious that many of the activities were mediated by the same proteins. For example, IL-2 had the activities associated with T cell growth factor, cytolytic T cell helper factor, and thymocyte mitogenic factor among others. Purified human GM-CSF was found to be responsible for the activity associated with neutrophil migration inhibitory factor (Wong *et al.*, 1985). Perhaps the most extreme example, however, has been IL-3 which has been shown to mediate a variety of activities associated with factors which, in many cases, would appear to be unrelated (Ihle *et al.*, 1983a). The spectrum of activities associated with IL-3 has however helped to better understand the function of IL-3 in stem cell differentiation.

The initial demonstration that IL-3 could mediate multiple activities came from studies of highly purified IL-3 (Ihle *et al.*, 1982b, 1983a). In these studies a reference stock of IL-3 was purified and characterized biochemically including establishing the sequence of the protein. This preparation was then used in a variety of assays and the dose–responses compared. In subsequent experiments an IL-3-specific antiserum was used to compare the inhibition of the various activities (Bowlin *et al.*, 1984). More recently, conditioned media produced by primate cells transfected with an expression vector containing an IL-3 cDNA clone has been shown to have a comparable spectrum of biological activities (Rennick *et al.*, 1985). Therefore there is little doubt that IL-3 mediates the activities described below. However, in many cases it is unclear whether other proteins may have comparable activities and in some cases the use of complex cell populations precludes determining whether the measured response is due directly to IL-3.

IL-3 was initially purified to homogeneity based on its ability to induce the enzyme 20αSDH in cultures of *nu/nu* splenic lymphocytes (Ihle *et al.*, 1982a). In a variety of studies the ability to induce 20αSDH in these cultures was shown to copurify from T cell conditioned media with IL-3 and was shown to be completely inhibited by antisera against IL-3 (Bowlin *et al.*,

1984) indicating that among a variety of factors IL-3 could uniquely induce 20αSDH in these cultures. In recent studies it has been reported that purified GM-CSF could also induce 20αSDH (Hapel *et al.*, 1985). These results are inconsistent with the previous studies and the possible basis for the differences were not considered. In the studies of Hapel *et al.* the purification and biochemical characterization of the GM-CSF used were not described since the preparation was obtained from another laboratory. In the enzyme assays presented the amount of enzyme activity was not proportional to the number of cells used and therefore errors in the assays may have contributed to the results. Irrespective, using highly purified T cell-derived GM-CSF we have been unable to substantiate the results.

From a technical standpoint the most useful activity associated with IL-3 is an activity termed WEHI-3 growth factor activity or hematopoietic growth factor activity (Ihle *et al.*, 1982a). These activities were ascribed to a factor which was present in WEHI-3 conditioned media and was required for the growth of a series of cell lines derived from long-term bone marrow cultures (Greenberger *et al.*, 1979; Dexter *et al.*, 1980). The assays involve a rapid and sensitive proliferation assay which is more convenient than the assays measuring 20αSDH induction. The availability of cell lines that require IL-3 for growth also has facilitated the initial characterization of receptors for IL-3 (Palazynski and Ihle, 1984) and has been an advantage in studies directed at the cell biology of IL-3 regulated growth.

The potential role of T cell-derived factors in mast cell growth and differentiation was first indicated by the observation that nematoid infections in athymic mice lacked the characteristic mast cell component observed in infections in normal mice (Ruitenberg and Elgersma, 1976). Subsequently, a number of groups independently described the presence of a factor in T cell conditioned media that was capable of inducing mast cell proliferation and differentiation (Yung *et al.*, 1981; Nagao *et al.*, 1981; Razin *et al.*, 1981; Nabel *et al.*, 1981; Schrader *et al.*, 1982a). In general it was observed that when bone marrow cells were cultured in conditioned media, the cells which persisted after 3–4 weeks in the cultures were mature mast cells and that their continued growth *in vitro* required a factor present in the conditioned media. This factor activity was generally referred to as a mast cell growth factor although the term persisting, cell-stimulating factor (PSF), was given to an identical activity. From a variety of studies it appears that in all cases the activity can be shown to be due to IL-3 and currently there is no evidence that another factor exists which is a growth factor for mast cells.

In a different approach to the study of mast cell differentiation, Dy *et al.* (1981) described a factor, histamine-producing cell-stimulating factor, which increased the histamine synthesis in cultures of normal spleen or bone marrow cells. Purified IL-3 has been shown to have this activity (Ihle *et al.*,

1983a). In associated studies, a factor was defined which induced increases in arginase levels in cultures of bone marrow or spleen cells (Dy *et al.*, 1983). This activity is also observed with IL-3 (Schneider *et al.*, 1985). Exactly what cell types are induced by IL-3 to express arginase, however, has not been determined.

The potential role of IL-3 in regulating early hematopoietic stem cell differentiation came from the observation (Goldwasser *et al.*, 1983) that IL-3 had an activity that had been ascribed to a factor termed burst-promoting activity (BPA). The concept of BPA came initially from the studies of Iscove *et al.* (1982) in which it was observed that a factor existed which could increase the number of erythroid bursts when added to bone marrow colony assays containing erythropoietin. From these studies it was proposed that the factor was acting by expanding the early precursors that could subsequently differentiate and become committed to the erythroid lineage. The fact that the factor came from T cells indicated that it may play an important immunological role in the regulation of hematopoietic stem cell differentiation. In several studies IL-3 has been demonstrated to have burst-promoting activity (Goldwasser *et al.*, 1983; Wagemaker and Ihle, unpublished data; Axelrad and Ihle, unpublished data). In the published studies of BPA the biochemical characteristics are consistent with the hypothesis that the factor is IL-3.

In studies of the regulation of the precursor cells for the monocyte–macrophage lineage capable of responding to CSF-1, the factor hemopoietin-2 was described (Bartelmez *et al.*, 1985). This factor supports the differentiation of cells *in vitro*, which acquire receptors for CSF-1 and can undergo terminal differentiation in the presence of CSF-1. In this regard the concept behind hemopoietin-2 is similar to the concept of the mechanism of BPA. Homogeneous IL-3 has been shown to have hemopoietin-2 activity; however, whether only IL-3 has this activity is not known. In additional studies (Koike *et al.*, 1986), IL-3 has been shown to synergize CSF-1 in the formation of macrophage colonies similar to the synergy which is observed between IL-3 and erythropoietin.

Purified IL-3 has been shown to induce the formation of colonies in soft agar cultures of bone marrow cells (Ihle *et al.*, 1983a; Prystowsky *et al.*, 1984) and therefore has the activity ascribed to colony-stimulating factors. The observation that the colonies induced by IL-3 morphologically contain a variety of lineages of cells has resulted in the use of the term multi-CSF for IL-3. Whether IL-3 is identical to previous factors termed multi-CSF, however, is unclear since these factor(s) have not been purified to homogeneity. For this reason it may be premature to assume that the multi-CSF activity from all sources is due to IL-3.

In recent studies "CSF" activities from induced murine T cell lines have

been characterized by using the IL-3-dependent cell lines described above and colony assays (Prestidge *et al.*, 1984). Based on the chromatographic properties of the activities, the term CSF-2α, CSF-2β, and CSF-2γ were proposed. From the description of the column systems used and the biological activities it would appear that CSF-2α and CSF-2β correspond to IL-3 and CSF-2γ correspond to GM-CSF. Although the nomenclature would suggest that unique factors are involved, there is little evidence to support this until the factors are further purified and are shown to be distinct from IL-3 or GM-CSF.

Perhaps one of the most interesting and important activities associated with IL-3 is the ability to induce the expression of Thy-1 in cultures of normal bone marrow cells or in cultures of *nu* splenic lymphocytes (Ihle *et al.*, 1983a). This type of activity was described for a factor, produced by activated T cells, which was termed Thy-1-inducing factor. In studies by Schrader *et al.* (1982) it was demonstrated that conditioned media from mitogen-stimulated T cells, when added to cultures of bone marrow, resulted in the appearance of 20–40% strongly Thy-1-positive cells after 3–4 days. Subsequent studies have demonstrated that among the hematopoietic growth factors and the lymphokines produced by activated T cells, the ability to induce Thy-1 in these cultures is uniquely associated with IL-3 (Keller *et al.*, 1985). The significance of this activity is considered in detail below.

In cultures of bone marrow cells, IL-3 has been shown to promote the differentiation or *in vitro* expansion of cells displaying a cytolytic activity associated with cells having natural cytotoxicity (NC activity) (Djeu *et al.*, 1983; Lattime *et al.*, 1983). This activity is distinguishable from NK cytolytic activity in a number of ways including differences in the cell surface phenotype, the spectrum of potential targets for lysis, and the ability of interferon to enhance the activity. Among the T cell-derived factors the ability to enhance NC activity appears to be a unique property of IL-3. The significance of this activity, however, is not clear and whether it is associated with specific lineages of IL-3-regulated cells is not currently known.

In recent studies IL-3 has been shown to augment a secondary anti-influenza cytolytic T cell response in collaboration with other helper factors including IL-2 (Curtsinger and Fan, 1984). The culture systems used, however, are complex and therefore it is difficult to determine whether IL-3 acts directly on cytolytic T cells or may be functioning through its affects on other cells and their ability to either produce additional factors or in an unspecified way to affect the activity. As discussed below, there is currently no evidence that IL-3 directly affects mature T cells.

Taken together, the above observations demonstrate that the addition of IL-3 to cultures of hematopoietic and lymphoid cells can have a variety of effects. As detailed below, one of the important goals is to understand how

many of the effects are related and can be accommodated in a model of IL-3 regulated growth and differentiation.

VI. Regulation of Hematopoietic/Lymphoid Stem Cell Differentiation

The spectrum of activities associated with IL-3 is consistent with a role in the regulation of early hematopoietic/lymphoid stem cell differentiation. In this section several aspects of potential roles for IL-3 in stem cell differentiation will be considered. From our perspective, one of the interesting questions to be resolved is the role of IL-3 in early T cell differentiation. In particular, does IL-3 promote the proliferation of cells which can become committed to the T cell lineage? Second, the question of the relationship of the differentiation of various lineages will be addressed and the available data relative to the sequence of differentiation promoted by IL-3 will be considered. Finally, since IL-3 is predominantly a T cell-derived lymphokine, the question arises as to whether IL-3 plays a central role in hematopoietic/lymphoid differentiation, or is an immunological mechanism for modulating stem cell differentiation.

In vivo reconstitution experiments have demonstrated that the bone marrow contains pluripotential stem cells which can differentiate along the myeloid and erythroid lineages as well as along T and B cell lineages (Wu *et al.*, 1968; Abramson, 1977). With regard to the T cells, little is known about the factors which regulate the proliferation and differentiation of bone marrow stem cells which will become committed to the T cell lineage. Once committed, however, the continued differentiation of T cells has been shown to be dependent upon migration to the thymus (reviewed in Stutman, 1978). In the absence of the thymus, the ontogeny of T cells is not observed. The function of the thymus in differentiation is not known but has been postulated to be required for the production of factors necessary for differentiation of the cells or for the selection of cells which have undergone appropriate T cell arrangements and express appropriate antigen recognition elements.

One approach to studying the factors that regulate the differentiation of bone marrow precursors for the T cell lineage is to study the regulation of the expression of early T cell markers. For this reason we have been studying the factor(s) which regulate the expression of Thy-1 and 20αSDH. In the initial studies of the tissue distribution of 20αSDH, the enzyme was found to be predominantly associated with mature T cell populations (Weinstein, 1977; Weinstein *et al.*, 1977; Pepersack *et al.*, 1980). In peripheral lymphoid tissues the majority of 20αSDH activity was found to be associated with Thy-1$^+$ cells. Consistent with the T cell origin, splenic lymphocytes from athymic mice or neonatally thymectomized mice were found to have little 20αSDH activity. More recently a variety of cloned T cell lines have been

shown to express high levels of the enzyme (Weinstein, Keller, and Ihle, unpublished data). As discussed below however, cells of other lineages may also express 20αSDH either transiently during differentiation or in mature cells.

The significance of the expression of 20αSDH by T cells is not known. 20αSDH reduces progesterone which inhibits T cell growth whereas the hydroxyl derivative is biologically inactive. One possible role for the enzyme therefore is to protect T cells from high concentrations of progesterone such as are found during late pregnancy. Interestingly, although 20αSDH is a marker for T cells in mice, T cells of other species including rats do not express the enzyme (unpublished data). Therefore the possibility exists that progesterone metabolism is only a cross-reactive activity and the real substrate has not been identified.

Within the thymus, 20αSDH is uniquely associated with the hydrocortisone-resistant, PNA-nonagglutinated Lyt-1^+,2^- population of medullary thymocytes (Pepersack, *et al.*, 1980; Keller *et al.*, 1985). This population is phenotypically and functionally similar to peripheral T cells and has been speculated to be the immediate precursors for peripheral T cells (for reviews see Scollay and Shortman, 1983; Scollay *et al.*, 1984; Mathieson and Fowlkes, 1984). In contrast, the hydrocortisone-susceptible, PNA-agglutinated population of corticol thymocytes do not express 20αSDH. This population, however, uniquely expresses the enzyme terminal deoxynucleotide transferase (TdT) (Coleman *et al.*, 1974; Barton *et al.*, 1976). Consistent with the *in vivo* distinction of TdT^+, $20\alpha SDH^-$, and TdT^-, $20\alpha SDH^+$ phenotypes, a variety of T cell lines, and T cell leukemias have also been shown to express one or the other of the two enzymes but rarely both (Pepersack *et al.*, 1980). To date we have only observed one exception (Profitt and Ihle, unpublished data).

The observation that *nu/nu* splenic lymphocytes lacked 20αSDH activity (Weinstein *et al.*, 1977; Ihle *et al.*, 1981a) but contained precursors for the T cell lineage (Gillis *et al.*, 1979) suggested that *in vitro* cultures might be used to study the factors which regulated the differentiation of cells capable of expressing 20αSDH. As discussed above, this assay was used to initially define IL-3. The term IL-3 was proposed to reflect the predominantly T cell origin of IL-3, the apparent relationship of 20αSDH to the T cell lineage, and to be consistent with the recommended nomenclature for T cell factors (Letter to the Editor, *Journal of Immunology* **123**, 2928, 1979).

The distribution of precursor cells, which can be induced to express 20αSDH by IL-3, has been examined (Keller *et al.*, 1985). A summary of the results are shown in Table II. In addition to splenic lymphocytes from *nu/nu* mice, splenic lymphocytes from normal mice contain a comparable population of cells. The highest frequencies of precursors in most strains of mice are in the bone marrow and are predominantly found in a large blasting fraction

TABLE II
INDUCTION OF PROLIFERATION AND 20αSDH IN VARIOUS HEMATOPOIETIC/LYMPHOID TISSUES BY VARIOUS FACTORS

Cell population	Assay	IL-3	IL-2	GM-CSF	CSF-1	G-CSF
PNA$^+$ thymocytes	20αSDH	−	−	−	−	−
	proliferation	−	−	−	−	−
PNA$^-$ thymocytes	20αSDH	−	+	−	−	−
	proliferation	−	+	−	−	−
Mature T cells	20αSDH	−	+	−	−	−
	proliferation	−	+	−	−	−
Bone marrow cells	20αSDH	+	−	−	−	−
	proliferation	+	−	+	+	+
Fetal liver cells	20αSDH	+	−	−	−	−
	proliferation	+	−	+	+	+

of cells that can be isolated by equilibrium sedimentation in gradients of bovine serum albumin (Keller and Ihle, unpublished data). This population has also been shown to be enriched for thymic precursors (Basch and Kadish, 1977) and to express high levels of 20αSDH. In both the spleen and the bone marrow, the IL-3-responding population is initially Thy-1 but, as described below, is induced to express Thy-1$^+$.

With thymocytes there is no detectable response to IL-3 in either assays for proliferation or for the induction of 20αSDH. The inability of the PNA-nonagglutinated, 20αSDH$^+$ cells to respond to IL-3 is consistent with the lack of an effect of IL-3 on cloned lines of mature T cells (Ihle *et al.*, 1982a) and the absence of receptors for IL-3 on these cells (Palasynski and Ihle, 1984). PNA-nonagglutinable T cells can, however, be readily expanded *in vitro* by activation with mitogen and culturing in the presence of IL-2. Under these conditions the cells continue to express high levels of 20αSDH (Keller *et al.*, 1985).

In addition to the major populations of the thymus as defined above, more recent studies have succeeded in identifying a minor population which has properties of a progenitor for the major populations (Scollay *et al.*, 1984). This population expresses Thy-1 and is characterized by the lack of expression of either Lyt-2 or L3T4. These cells are present early in the ontogeny of thymocytes and approximately 30–50% of the cells express receptors for IL-2 as assessed by monoclonal antibodies (Boehmer *et al.*, 1985; Raulet, 1985; Ceredig *et al.*, 1985). Although expressing IL-2 receptors, the cells do not proliferate to IL-2. Whether these cells would proliferate to IL-3 is not known. However, IL-3 has been found to induce proliferation in

cultures of thymocytes from newborn mice in contrast to adult mice (Keller, unpublished data).

The lack of a response of the PNA agglutinated, 20αSDH$^-$ subpopulation has considerable significance. In some models of T cell differentiation, it has been proposed that the thymic, corticol population of PNA-agglutinated, TdT$^+$, 20αSDH$^-$ cells differentiates within the thymus to the more mature population of thymocytes. For this reason it might have been anticipated that IL-3 would induce the differentiation of the corticol population to express 20αSDH. This activity has not been detected with IL-3 or with IL-2 or with unfractionated conditioned media from activated T cells. In addition, none of the thymic factors can induce proliferation or maturation of corticol thymocytes (Andrews *et al.*, 1985). Therefore, either unknown factors, possibly of thymic origin, have this activity, the frequency with which this differentiation occurs is too low to measure, or there is not a simple developmental relationship between the two major thymus subpopulations.

In support of independent origins, a number of studies have demonstrated that the corticol population is a "sterile" population of thymocytes and that the majority of the cells never leave the thymus (McPhee *et al.*, 1979; Scollay and Weissman, 1980; Shortman and Jackson, 1974). The unique expression of TdT by this population of thymocytes suggests that TdT may be involved in this property. Consistent with this hypothesis conditions that stimulate high TdT synthesis *in vitro* are not associated with mitogenesis but rather with growth arrest (Rothenberg and Triglia, 1983).

The function of thymocytes expressing TdT$^+$ is not known but it has been proposed that they are T cells in which *V* gene rearrangements have given rise to potentially autooreactive cells and which are undergoing clonal deletion in the thymus (Ihle *et al.*, 1981a; Bollum, 1981; Ihle and Keller, 1984). In this model, *V* gene rearrangements occur during commitment of cells to the T cell lineage within the bone marrow. If rearrangements give rise to T cell receptors that recognize self, the cells are induced to express TdT whereas rearrangements that give rise to receptors which are not autoreactive differentiate along a pathway in which 20αSDH is expressed. In this model it is speculated that the specific function of TdT is to assure cell death by its mutagenic effects during DNA replication.

In addition to its speculated role in early T cell differentiation, the spectrum of activities associated with IL-3 demonstrates that it regulates an early hematopoietic stem cell which is capable of becoming committed to a variety of other lineages. In cultures of bone marrow cells in IL-3, morphologic evidence exists for the differentiation of a variety of lineages of hematopoietic cells (Prystowski *et al.*, 1984). The predominant cell types during the initial 2 weeks of culture are granulocytes and macrophages. Subse-

quently the major cell type in the cultures becomes mast cells. The relationship between the various cell types is not precisely known but studies of IL-3-induced Thy-1$^+$ cells have helped to begin to understand the possible relationships (below).

The general ability of IL-3 to support the proliferation of pluripotential stem cells has come from the observation that IL-3 can support the growth *in vitro* of cells which can give rise to spleen colony-forming cells (CFU-S) (Spivak *et al.*, 1986). In *in vitro* cultures IL-3 prevented the loss of CFU-S activity observed in media alone. However, IL-3 could not maintain CFU-S for longer than 10–12 days in culture. The cells responding to IL-3 from bone marrow were found to be initially Thy-1$^-$ whereas after culture approximately half of the CFU-S activity was associated with a Thy-1$^+$ cell, suggesting that IL-3 promotes the differentiation of cells *in vitro* and that the phenotypic characteristics of colony-forming cells vary with differentiation.

As noted above, IL-3 has been shown to be the only known hematopoietic/lymphoid growth factor to induce the expression of Thy-1 in cultures of bone marrow cells. In these cultures Thy-1 is induced over the first week of culture and is expressed at levels that are comparable to the levels observed on thymocytes. Using a fluorescence-activated cell sorter, the cells which have been induced for Thy-1 expression have been isolated and the properties studied (Keller *et al.*, 1985; Keller and Ihle, 1986). A summary of the properties of the Thy-1$^+$ cells is given in Table III. The Thy-1$^+$ cells express high levels of 20αSDH and proliferate *in vitro* to IL-3. In colony assays approximately 1 in 700 cells can form colonies whereas in single cell cultures 1 in 2–3 cells can proliferate. Although the expression of both 20αSDH and Thy-1 is associated with T cells, no response can be shown of the Thy-1$^+$ cells to IL-2 in the presence or absence of Con A. In contrast, the majority of the cells can proliferate in response to the T cell-derived lymphokine GM-CSF. In the absence of added growth factors, no proliferation is observed and the viability of the cells is rapidly lost.

On continued culture, the Thy-1$^+$ cells differentiate and give rise to mature granulocytes and macrophages during the first week. With long-term culture, homogeneous populations of mast cells are obtained. During this differentiation Thy-1 expression if rapidly lost such that after 2 weeks fewer than 5% of the cells continue to express Thy-1 demonstrating that the expression of Thy-1 is only transient during differentiation. Similarly the expression of 20αSDH is transient and the levels of enzyme activity decrease with time in culture. In these cultures, the differentiation of granulocytes and macrophages will occur when the Thy-1$^+$ cells are cultured in either IL-3 or GM-CSF whereas the differentiation of mast cells occurs only in the presence of IL-3.

TABLE III
CHARACTERISTICS OF Thy-1$^+$ AND Thy-1$^-$ CELLS FROM IL-3 SUPPLEMENTED CULTURES OF BONE MARROW

Property	Thy-1$^+$	Thy-1$^-$
20αSDH pmol/hour/10^6 cells	40–120,000	4–17,000
Stimulation index IL-3	7.8	9.5
CFU/10^6 cells IL-3	1500	115
Frequency responders IL-3	1/2–3	<1/100
Stimulation index GM-CSF	3.6	2.9
CFU/10^6 cells CSF	635	7
Frequency responders GM-CSF	1/2–3	<1/100
Stimulation index IL-2	1.2	1.1

Relative to the transient expression of Thy-1 is the question of when during differentiation do individual cells become committed to the various lineages. This aspect has been addressed by studying the progeny of individual Thy-1$^+$ cells cultured in IL-3. The results demonstrate that individual Thy-1$^+$ cells can give rise to either homogeneous populations of granulocytes or macrophages or can give rise to mixed colonies suggesting that it is during the time that the cells are expressing Thy-1 that the commitment to various lineages is occurring. In these studies, the commitment to the mast cell lineage could not be addressed due to the low frequency with which cells become to committed to this lineage. Moreover, whether it is during the expression of Thy-1 that cells become committed to the T cell lineage has not been addressed.

From studies of the type described above, a scheme for IL-3-induced differentiation has been developed which is shown in Fig. 4. An IL-3-responsive "stem cell" exists which is primarily found in the bone marrow but

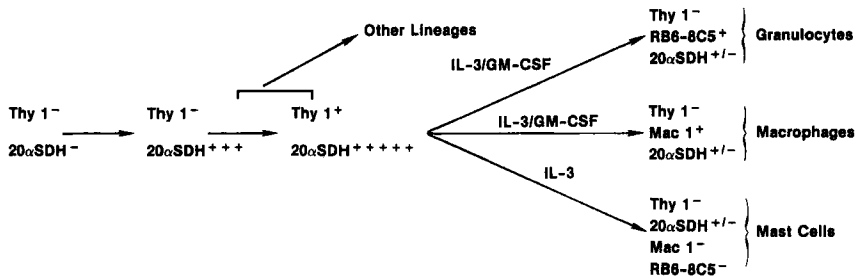

FIG. 4. A proposed scheme for IL-3-induced differentiation.

which is also present in fetal liver and in adult spleens. In the bone marrow the precursor is noncycling and in a stochastic manner acquires the ability to respond to IL-3 (Suda *et al.*, 1985). At this point the continued viability of the cell appears to be dependent on the presence of IL-3. The earliest detectable response to IL-3 is the induction of the expression of 20αSDH and subsequently the cells are induced to express Thy-1. Whether all cells go through an intermediate which expresses Thy-1 is not known. During the expression of Thy-1, the cells become committed to various lineages including granulocytes, macrophages, and mast cells. It is hypothesized that cells may also become committed to the T cell lineage although no direct proof exists for this.

The observation that IL-3-induced cells, which are committed to the myeloid lineage, respond to GM-CSF suggests that the cells acquire the ability to respond to GM-CSF as a consequence of differentiation. Whether this involves the induction of the expression of receptors for GM-CSF by IL-3 is not known. As indicated, continued differentiation in the presence of either IL-3 or GM-CSF results in the loss of expression of both Thy-1 and 20αSDH. With differentiation the cells lose the ability to proliferate and following terminal differentiation the cells die out. In contrast, with terminal differentiation mast cells retain the ability to proliferate for extended periods *in vitro* in the presence of IL-3. It is this property which accounts for the presence of homogeneous populations of mast cells in long-term bone marrow cultures in IL-3.

The concept that cells acquire the ability to respond to various hematopoietic growth factors with differentiation may apply to other lineages. In particular, IL-3 increases the number of cells capable of differentiating in the presence of erythropoietin (Goldwasser *et al.*, 1983). Whether this cell is derived from the Thy-1$^+$ population has not been addressed. Nevertheless, it is likely that IL-3 supports the differentiation and commitment of progenitors to the erythroid lineage. In this case, however, the continued differentiation of the cells is dependent on the presence of a lineage-specific growth factor. In this regard it should be noted that in one study it was observed that IL-3 alone could support the terminal differentiation of erythrocytes (Goodman *et al.*, 1985). This has not been a reproducible finding. The available evidence also suggests that IL-3 induces the differentiation of cells which acquire receptors for CSF-1 (Bartelmez *et al.*, 1985; Koike *et al.*, 1986). Finally, it can be speculated that the cells which become committed to the T cell lineage may become dependent on the thymic microenvironment for continued differentiation.

The significance of the expression of Thy-1 in IL-3-promoted differentiation is not known although the expression of low levels of Thy-1 on high proliferative, pluripotential stem cells has been observed in a several stud-

ies. Initially Thierfelder (1977) demonstrated that in rats Thy-1 was a specific marker for hematopoietic stem cells and subsequent studies confirmed these results (Goldschneider, 1980) and further indicated that pre-B cells may also express Thy-1 (Crawford and Goldschneider, 1980). Thy-1 is not expressed on rat mature B or T cells nor is it expressed on mature myeloid cells. Thy-1 is expressed, however, in the thymus and in the bone marrow where approximately 30–45% of the cells express Thy-1 (Williams, 1976). In mice low levels of Thy-1 are detectable on hematopoietic stem cells (Basch and Berman, 1982; Schrader *et al.*, 1982; Boswell *et al.*, 1984; Miller *et al.*, 1985). The levels observed are considerably lower than the level of expression seen with thymocytes or with bone marrow cells which have been induced to express Thy-1 with IL-3 *in vitro*. Whether the levels of expression may be due to the rate of proliferation or stage of differentiation has not been addressed.

In other species the expression of Thy-1 varies considerably. In syrian hamsters the Thy-1 homolog is expressed on both peripheral T and B cells (Witte and Streilein, 1983). In humans and dogs the Thy-1 analog is not expressed on peripheral T cells (Dalchau and Fabre, 1979). Dog thymocytes express only low levels of Thy-1 and Thy-1 is not detectable on human thymocytes. Whether Thy-1 is expressed on hematopoietic/lymphoid stem cells in these species is not known. Irrespective, the results suggest that when Thy-1 expression is induced may be of more importance in understanding the function of Thy-1 than when and in what lineages its expression is lost with differentiation. This suggests the possibility that Thy-1 expression may have relevance for cellular interactions of differentiating stem cells in the bone marrow. One highly speculative possibility is that expression of Thy-1, since it appears to occur on cells undergoing lineage restrictions, may signal cells to migrate out of the bone marrow.

One of the important considerations in hematopoietic/lymphoid differentiation is the concept of cycling of stem cells. Although still preliminary the available data do not indicate that IL-3 supports self-renewal but rather once a stem cell becomes responsive to IL-3, it is committed to differentiate. A central question therefore is the origins of the factors which regulate the precursors for the IL-3-responsive cell. As noted below, it may be possible to study this aspect by using long-term bone marrow culture systems.

Although IL-3 supports the proliferation and differentiation *in vitro* of granulocytes and macrophages, there is some question that IL-3 is obligatorially associated with myeloid differentiation. For example, the Dexter culture systems support normal granulopoiesis *in vitro* (Dexter *et al.*, 1977) in the absence of detectable IL-3 or IL-3-associated differentiation as assessed by the presence of Thy-1$^+$ cells, expression of 20αSDH, or the presence of mast cells. Interestingly, however, the system maintains precur-

sors which can respond to IL-3. This is the likely basis for reports of cells in Dexter cultures that could be induced to express Thy-1 with conditioned media from mitogen-stimulated T cells (Jones-Villeneuve and Phillips, 1980). In addition, it has been shown that the Dexter culture system maintains precursors which in irradiated recipients can give rise to lymphocytes (Schrader and Schrader, 1978; Jones-Villeneuve and Phillips, 1980). In more recent studies using mice with a recessive mutation leading to a severe combined immune deficiency (scid), it has been shown that long-term cultures also maintain precursors for B cells as well as T cells (Phillips *et al.*, 1984).

In vivo there is also evidence that IL-3 may not be involved in normal hematopoiesis. During development the fetal liver is a major site of myeloid differentiation but does not contain cells capable of producing IL-3 or evidence of IL-3-promoted differentiation as determined by the expression of Thy-1 or 20αSDH. In *nu/nu*, athymic mice hematopoiesis is normal although these mice lack a detectable source of IL-3. Similarly, scid mice lack mature T cells as well as B cells, but have normal hematopoiesis (Bosma *et al.*, 1983). From these observations it can be proposed that there may exist a "constitutive" pathway of myeloid differentiation which does not involve IL-3. IL-3-associated differentiation is uniquely characterized by the expression of 20αSDH and Thy-1. In addition, IL-3-associated differentiation may be characterized by a broader spectrum of potential lineages including T cells and mast cells.

A number of interesting aspects associated with IL-3-induced differentiation remain to be resolved. The most important question relative to our interests is whether cells, during the period of Thy-1 expression, become committed to the T cell lineage. Experiments are currently in progress to directly address this aspect. The existence of a common progenitor for lymphoid and myeloid cells has been suggested by the properties of leukemias such as myelogenous leukemias which have a lymphoblastoid phase (Boggs, 1974) and leukemias with mixed lymphoid and myeloid characteristics (McGraw *et al.*, 1981; Jehn and Thiel, 1981). More recently the conversion of a T cell to a myeloid leukemia was documented (Hershfield *et al.*, 1984). In addition, studies of hematopoietic cell differentiation in mice have suggested a close lineage relationship between myeloid and T cells (Wu *et al.*, 1968; Abramson, 1977; Fleischman *et al.*, 1982).

The ontogeny of the ability to produce IL-3 and the expression of 20αSDH are consistent with a possible role in T cell differentiation. In particular cells expressing 20αSDH appear late in fetal development with a rate of appearance comparable to the expression of Thy-1 and the expression of T cell receptors. Shortly after birth there is large increases in 20αSDH in both the

thymus and in the spleen. In addition there is a rapid increase in the frequency of peripheral T cells which can be induced to secrete IL-3 as well as IL-2 and GM-CSF. It should be noted that this pattern of ontogeny is quite distinct from the ontogeny of B cells which generally occurs much earlier. How these observations are related and the significance to understanding the regulation of the normal differentiation of T cells is not known.

Whether IL-3 also regulates the differentiation of cells which can become committed to the B cell lineage is not known. In general, B cell differentiation is not known to be associated with either the expression of Thy-1 or 20αSDH. In recent studies, however (Palacios *et al.*, 1984), a series of cell lines were described which had the phenotypic properties of early B cells and which required IL-3 for growth *in vitro*. In addition, the lines contained rearrangements of the heavy chain locus and the κ light chain locus and expressed low levels of cytoplasmic immunoglobulin. Under appropriate conditions the cells could be induced to differentiate *in vitro* and to secrete IgM. In these regards the cell lines are distinct from a variety of IL-3-dependent cell lines obtained in other laboratories. For this reason, the lines are not representative and therefore may not constitute normal intermediates in IL-3-promoted differentiation.

VII. Potential Mechanisms for Interleukin 3 Regulation *in Vivo*

The broad spectrum of activities associated with IL-3 *in vitro* suggests that *in vivo* IL-3 may mediate a variety of aspects of immune responses. Conceptually IL-3 can be envisioned to mediate effects in the bone marrow where it could increase the number of proliferating stem cells which are committing to various lineages. Second, IL-3 may function at peripheral sites of antigen activation of T cells to allow the continued proliferation and differentiation of stem cells or committed progeny. Among the functional progeny, only immune mast cells are known to uniquely continue to require IL-3 for proliferation suggesting that this lineage may provide a marker for the production of IL-3.

Any consideration of the potential *in vivo* activities of IL-3 must take into account the levels of IL-3 that are observed. The ability to produce sustainable circulating levels of IL-3 would suggest that proliferation and differentiation of stem cells will occur systemically. If, however, circulating levels of IL-3 are rarely attained the effects of IL-3 are likely to be more localized. In the latter situation it is relatively easy to envision sites of activation of T cells in infections and the local production of IL-3 which together with GM-CSF and IL-2 contribute to the properties of inflammatory responses. How activation of T cells at peripheral sites might be translated to increased stem

cell proliferation in the bone marrow is less obvious, but may involve the selective localization of activated T cells to microenvironments in the marrow.

During immune responses there is generally not detectable circulating levels of IL-3. For example we have examined mice which were regressing MoLV/MSV induced tumors and mice which were viremic with MoLV and had helper T cells capable of producing IL-3 to MoLV gp70 *in vitro* (unpublished data). Circulating levels of IL-3 were also not detected in mice with severe graft-verses-host disease (GVDH) (Garland and Crompton, 1983). In these and other studies (Crapper *et al.*, 1984) the absence of activity in the serum was not due to the presence of inhibitors of IL-3 activity. In one system, however, it has been possible to transiently detect IL-3 in circulation (Filho *et al.*, 1983). In this example, mice were infected with *Nippostrogylus brasiliensis* and were challenged with an iv injection of adult worm antigen. Under these conditions IL-3 activity was detectable in the serum after 4 hours and decreased rapidly thereafter. Without introducing antigen, only small inconsistent levels of IL-3 were observed in the sera.

The absence of circulating levels of IL-3 under all but extreme immunological situations is comparable to that observed with IL-2. With IL-2, it had been proposed that a serum inhibitor existed (Hardt *et al.*, 1981) although this has not been a reproducible observation (Donohue and Rosenberg, 1983). In contrast to IL-3 and IL-2, circulating levels of GM-CSF have been observed in endotoxin-treated mice. Whether this activity is due specifically to activated T cells and is detectable in athymic mice has not been examined. It is possible that the extent of T cell activation required for attaining circulating levels of GM-CSF may be less than for IL-3 or IL-2 since activated T cells produce considerably more GM-CSF than IL-3.

The measured half-lives of IL-3 and IL-2 *in vivo* are comparable. In the case of IL-2 Donohue and Rosenberg (1983) measured a half-life of 3 minutes. Using partially purified IL-3 Garland and Crompton (1983) observed a serum half-life of less than 30 minutes. In similar studies the half-life of IL-3 was shown to be composed of an initially rapid turnover of approximately 4 minutes followed by a slower phase of approximately 40 minutes (Crapper *et al.*, 1984). Studies have not been done with highly purified IL-3.

Although in most cases circulating levels of IL-3 are not detected there are increases during immune responses in the frequencies of cells which can respond to IL-3. Perhaps the most dramatic increases that have been observed are those seen in mice infected with MoLV. In this system newborn mice are inoculated with virus and by 2–3 weeks of age virus is replicating in a variety of tissues and the mice become viremic. The animals subsequently develop leukemia with latencies of 3–6 months. In these mice, Lyt-1^+,2^- helper T cells are induced (Lee *et al.*, 1981) which *in vitro* produce lympho-

kines including IL-3, IL-2, and GM-CSF (Ihle *et al.*, 1981a; unpublished data). In CBA/n mice the helper T cells are not induced and these mice have either no leukemia (Lee and Ihle, 1981) or leukemia develops late (Storch and Chused, 1984). In BALB/c mice, in which helper T cells are induced, there is a 50- to 200-fold increase in the frequency of cells responding to IL-3 (Lee and Ihle, 1981) as well as to other lymphokines. These increases are dependent on the presence of helper T cells and do not occur in CBA/n mice.

Taken together, the above observations have been used to propose a model by which immune responses to retroviral antigens can contribute to leukemia. (Ihle *et al.*, 1984). In this model, it is proposed that chronic, systemic production of IL-3 occurs as a consequence of the viremia and the presence of viral antigen-reactive T cells. This results in the amplification of lymphokine-responsive populations. These proliferating cells are envisioned to be an essential "target" cell population for transformation by viral integration or somatic mutations or rearrangements. As noted below, this model is supported by the observation that a number of the tumors involve cells with properties of IL-3-regulated cells and in many cases continue to require IL-3 for growth *in vitro*.

Increases in the frequencies of lymphokine-responsive cells occur in normal immune responses but are generally of a lesser magnitude and duration than the above. During the regression of tumors induced by MoLV/MSV in C57BL/6 mice there is an increase in the frequencies of lymphokine-responsive cells in the spleen which peaks at the peak of tumor regression and subsequently decreases to normal levels within 20 days (Lee *et al.*, 1981). In mice immunized with sheep erythrocytes, the iv inoculation of sheep erythrocytes results in increases in the numbers of IL-3 responsive in the spleen (Crapper and Schrader, 1983; Crapper *et al.*, 1984). This does not occur in athymic mice indicating the dependence on T cells. In mice injected with keyhole limpet hemocyanin in one footpad, there was an increase in the frequency of IL-3-responsive cells in the regional lymph node but not in the contralateral lymph node.

Taken together, the limited *in vivo* data suggest that the activation of helper T cells results in the local amplification of IL-3-responsive cells. Relative to the functionally mature cells, the predominant effects seen are on mast cells because of their unique requirement for IL-3 for continued proliferation. This effect is comparable to the effects seen with IL-2 and the amplification of cytolytic T cells. In addition, however, IL-3 has the potential to cause the amplification of cells capable of differentiating along multiple lineages. Whether the increases seen in the frequencies of IL-3-responsive cells represent cells at this stage in differentiation is not known. In addition, it is not known whether in some situations the predominant effects of immu-

nological-regulated production of IL-3 are on stem cells in the bone marrow. Within the general subject of the effects of IL-3 *in vivo*, it should be reiterated that the production of IL-3 is inhibited by cyclosporin A. Therefore some of the immunosuppressive and possible antiinflammatory effects may be due to the inhibition of IL-3 production. Similarly the ability of glucocorticoids to inhibit IL-3 production may account for part of its antiinflammatory effects.

VIII. Interleukin 3-Responsive Cell Lines: Origins and Properties

Studies of the mechanisms by which IL-3 regulates the growth of hematopoietic stem cells have been greatly facilitated by the existence of a variety of cell lines which require IL-3 for growth. IL-3-dependent cell lines have been derived from three sources including long-term bone marrow cultures, cultures of mast cells, and primary retrovirus-induced leukemias. As noted above, the initial cell lines were developed by Greenberger *et al.* (1979) from long-term bone marrow cultures. The most widely distributed lines obtained from these studies are the 32-DCL and B6Sut lines. Using the procedures described by Greenberger *et al.* (1979), Dexter *et al.* (1980) also developed IL-3-dependent cell lines. The lines were termed FDC (factor dependent cells)-P (Patterson Laboratories) and the FDC-P1 and FDC-P2 lines have been widely distributed.

The mechanisms involved in the derivation of IL-3-dependent cell lines from long-term bone marrow culture systems are not known. In the initial studies (Greenberger *et al.*, 1979) it appeared that the frequency of establishing cell lines was higher when the cultures were infected with retroviruses. However, subsequent studies (Dexter *et al.*, 1980) did not confirm these results. There is, however, a peculiar requirement for subculturing from the long-term bone marrow cultures into WEHI-3 conditioned media. Cell lines are not obtained by culturing bone marrow cells directly into WEHI-3 CM. This may be significant because the initial bone marrow cultures do not contain IL-3, but can maintain cells which will differentiate in response to IL-3. The derivation of IL-2-dependent cell lines has been postulated to require transformation (Nabholz *et al.*, 1980; Gillis and Watson, 1981). It appears likely that the derivation of long-term cell lines from bone marrow cultures similarly involves some type of transformation.

The properties of a number of cell lines from long-term bone marrow cultures have been examined (Ihle *et al.*, 1982b; Garland, 1984). The expression of various cell surface markers has provided little indication of lineage. None of the lines expresses markers which are associated with the B cell lineage including 2C2 and ThB. This is in contrast to the recent studies of Palacios *et al.* (1984) who described an IL-3-dependent cell line which did

express markers associated with the B cell lineage. In general, the cell lines do not express markers associated with mature T cells including Lyt-1 or Lyt-2. However, many of the lines express Thy-1 at levels which are comparable to that found on thymocytes. In addition, all the cell lines have 20αSDH activity. A number of the lines, however, express Mac-1 and some contain nonspecific esterase activity. With regard to the phenotypes, therefore, it has not been possible to precisely define the lineages, although the general properties are like the IL-3-induced Thy-1$^+$ cells observed in normal differentiation and the expression of Mac-1 suggests, in some cases, a relationship to the myeloid lineage.

At least a few of the cell lines have been speculated to represent multipotential hematopoietic progenitors (Greenberger *et al.*, 1983). In *in vitro* cultures, at low frequencies, cells were shown to differentiate to erythroid cells, neutrophil granulocytes, and basophil/mast cells. In other studies a series of cell lines derived from long-term cultures appeared more restricted in their ability to differentiate and predominantly show limited differentiation to granulocytes. Taken together, the results suggest that the cell lines vary in their ability to differentiate and may represent cells at different stages of differentiation and committment.

In cultures of bone marrow in IL-3, the predominant cells which persist after 3–4 weeks have the functional and phenotypic properties of mast cells. In these cultures, mast cells will continue to proliferate for considerable periods of time and it is not unusual to maintain the cultures for 3–5 months. Occasionally, however, long-term, continuous cell lines have been obtained from these cultures which continue to require IL-3 for growth (Nabel *et al.*, 1981a; Nagao *et al.*, 1981; Schrader *et al.*, 1982; Sredni *et al.*, 1983). When characterized, these cells retained the functional and morphological properties of mast cells and were Thy-1$^-$ and where examined expressed 20αSDH (Ihle, unpublished data). Unlike a number of the IL-3-dependent cell lines, mast cell lines do not respond to GM-CSF (Ihle, unpublished data). In these cases, therefore, mature mast cells have been "immortalized" but are largely unaltered with regard to their differentiated phenotype or their requirement for growth factors. As discussed below, there are several murine-transforming viruses which can induce this phenotype.

A number of IL-3-dependent cell lines have been established from primary retrovirus-induced lymphomas (Ihle *et al.*, 1984; Holmes *et al.*, 1985; Oliff *et al.*, 1984). The rational for examining the ability of primary leukemias to grow *in vitro* in IL-3 has been reviewed in detail (Ihle *et al.*, 1984) and therefore will not be discussed. In the original studies, it was demonstrated that approximately 30% of the primary leukemias induced in BALB/c mice by Moloney leukemia virus could be grown *in vitro* if IL-3 was added to the culture medium. In subsequent studies (Oliff *et al.*, 1984), it was found that

leukemia cells from Friend MuLV-induced erythroleukemias could also be grown in culture in the presence of conditioned media from the WEHI-3 cell line. Although not demonstrated, it is likely that these cell lines are dependent upon IL-3.

In additional studies (Holmes *et al.*, 1985), primary tumors, induced by a wild mouse ecotropic, were examined for their growth properties *in vitro*. This system was interesting because the virus used (Cas-Br-MuLV) induces a spectrum of hematopoietic tumors (Frederickson *et al.*, 1984). Among these, approximately 80% of the myeloid and erythroid tumors could be established as cell lines *in vitro* in the presence of IL-3. In contrast, IL-3 did not support the growth of most of the thymic lymphosarcomas or B cell lymphomas. Morphologically, all the cell lines which were derived were myeloid in appearance and generally expressed Mac-1. All the myeloid leukemias also expressed Thy-1 and 20αSDH.

The properties of the myeloid leukemias have been studied with the goal of determining their relationship to normal IL-3-promoted differentiation (Ihle *et al.*, 1985, 1986; Weinstein *et al.*, 1986). The results suggest that the cells are comparable to IL-3-induced Thy-1$^+$ intermediates which have been committed to the myeloid lineage. In particular, unlike the sorted normal Thy-1$^+$ cells, the myeloid lines express high levels of 20αSDH and Thy-1 at levels which are comparable to that observed with T cells by either immunofluorescence or by Northern analysis of poly(A)-selected RNA. Like the normal Thy-1$^+$ cells, the continued proliferation of the cells is dependent on the presence of IL-3. In addition, the cells proliferated in response to GM-CSF, although this lymphokine has not been found to sustain long-term growth. Morphologically the cells are myeloblastic in appearance. Unlike the normal FACS-purified Thy-1$^+$ cells, however, there is no indication that the myeloid leukemia cell lines undergo differentiation in the presence of either IL-3 or GM-CSF or in the absence of growth factors. Therefore the properties of the cells are consistent with the concept that the cells are blocked in their ability to terminally differentiate. This has not, however, detectably altered the requirements or response to normal growth factors.

The potential genes that may be affected in the above myeloid leukemias are largely unknown. However, in a least one case, the inability to differentiate may be associated with a rearrangement of the c-*myb* locus. We initially examined for potential rearrangements of the c-*myb* locus based on the observation that in chickens this type of transformation is characteristic of the avian myeloblastosis virus (AMV) which contains the v-*myb* oncogene (Graf and Beug, 1978; Beug *et al.*, 1982). When a number of myeloid leukemias were examined, one (NFS-60) was found to have a genomic rearrangement of one of the alleles of the c-*myb* locus. Using cloned genomic fragments, the rearrangement was found to involve the insertion of a provirus in

the middle of the cellular gene (Weinstein *et al.*, 1986). By Northern analysis, the cells contained a truncated mRNA of approximately 2 kb relative to the normal mRNA of approximately 4 kb. Experiments are currently in progress to determine whether the rearranged allele can induce a comparable phenotype *in vitro* in cultures of normal cells differentiating in the presence of IL-3. The absence of comparable rearrangements in a number of phenotypically comparable cell lines suggests that other genes may affect the cells comparably.

It should be noted that there are striking similarities between the cell cells derived from primary leukemias and those obtained from the long-term bone marrow culture experiments. For this reason several of the long-term bone-derived lines have been examined for rearrangements of various oncogenes including the c-*myb* locus. In none of the cell lines have we detected rearrangements of c-*myb*, c-*myc*, c-*ras* (Ha, Ki, N), or c-*mos*. Unfortunately the transforming events involved in the induction of most myeloid leukemias are not known.

IX. Mechanisms in Interleukin 3-Regulated Growth

There have been relatively limited studies concerned with the cell biology of IL-3-dependent cells. In general, however, the properties of IL-3-regulated cell growth appear comparable to those of IL-2-regulated growth. In particular, like IL-2-dependent cells, IL-3-dependent cell lines require IL-3 for maintenance of viability as well as for the induction of proliferation. In studies of the rate of loss of viability of the IL-3-dependent cell lines, C3Hsffv, 32-DCL, and FDC-P1 it was observed that the cells lost the ability to recover by either measuring thymidine incorporation or by measuring cloning efficiency with biphasic kinetics (Ihle *et al.*, 1982a). During the first 6 hours, the cells lost relatively little viability in the absence of IL-3 and could recover with the readdition of IL-3. Subsequently, the cells began to die with apparent first-order kinetics such that half the cells lost viability every 1–6 hours depending on the cell line. In more recent studies, employing the 32-CL13 line, Metcalf (1985) has confirmed the above studies although the first phase was not observed. Comparable kinetics have been observed with IL-3-dependent cell lines derived by myeloid leukemias (Ihle *et al.*, 1985).

Whether IL-3 is required throughout the cell cycle has not been analyzed in detail. Studies with IL-2-dependent T cell lines suggest that IL-2 is required for the entry of cells into the S phase. In the absence of IL-2, the cells accumulate in G_1 (Brown *et al.*, 1982; Sekaly *et al.*, 1982). In comparable experiments we have evaluated the length of exposure that is required for IL-3 to induce one round of proliferation (Ihle, unpublished data). To

obtain 50% of maximum stimulation, IL-3 was required for 8 hours, suggesting that exposure to IL-3 throughout the G_1 phase is required. Following stimulation there was a peak of DNA synthesis which occurred between 12 and 16 hours. Once initiated, continued DNA synthesis was not dependent on the continued presence of IL-3. Taken together with the apparent lag in the loss of the viability of the cells in the absence of IL-3, the results suggest that IL-3 is primarily required during the G_1 phase to induce a commitment to proliferate. After this IL-3 is no longer required. These results are in contrast to studies by Metcalf (1985) which indicated that IL-3 was required at all times of the cell cycle. The differences may be due to the use of colony-forming assays in the latter studies.

IL-3-specific cell surface receptors have been detected on the factor-dependent cell lines including the 32D-cl and FDC-P1 cell lines (Palasynski and Ihle, 1984). In contrast, IL-3-specific receptors have not been detected on a variety of cell lines which do not require IL-3 for growth including IL-2-dependent cell lines. A K_d of 5.4×10^{-11} and a maximum binding capacity of 6.25 fmol per 10^6 cells was calculated for 32D-cl 23 cells from equilibrium binding studies using ^{125}I-labeled IL-3. A similar analysis for the FDC-P1 cells yielded a K_d of 1.7×10^{-11} and a maximum binding capacity of 1.2 fmol per 10^6 cells. The data suggest that the 32D-cl 23 cells contain approximately 4000–5000 high-affinity binding sites/cell whereas the FDC-P1 cells have 1500–2500 sites/cell. The binding was specific for IL-3 and, in particular, factors such as IL-2 or GM-CSF did not compete for the binding. These results suggest, therefore, that the ability of myeloid leukemia cell lines to proliferate in response to both IL-3 and GM-CSF is due to the presence of unique receptors for each of the growth factors.

A comparison of the biological response curves for iodinated IL-3 with the binding curves demonstrates that biological activity is observed over the range of concentrations of IL-3 that are required for binding. This observation supports the hypothesis that the biological activity is mediated by binding to the receptors. There was, however, a slight displacement between the binding curves and the curves for induction of biological responses. This was speculated to be due to the ability to induce a maximal biological response by binding of IL-3 by only a fraction of the available receptors. This type of relationship has been observed for other growth-promoting factors (Cuatracasas and Hollenberg, 1976).

The mechanisms by which IL-3 interacts with its receptor function to induce proliferation of the cells are not known. In studies employing the FDC-P2 cell line (Whetton and Dexter, 1983), it was observed that an ATP-generating system composed of ATP, creatine phosphate, and creatine phosphokinase could replace the requirement for IL-3. The important component in the system was shown to be ATP, suggesting that IL-3 may mediate its effects on growth by regulating intracellular ATP levels. Consistent with

this concept, in the absence of IL-3, intracellular ATP levels decreased and could be restored to normal by exogenous ATP or by the addition of IL-3.

In a subsequent study Palacios and Garland (1984) demonstrated that one IL-3-dependent cell line (S-480-3) of basophils responded to exogenous ATP but another cell line (Ea3) did not respond to ATP. From these results it was concluded that two mechanisms may be involved in growth regulation. One is speculated to involve a ligand–receptor-mediated mechanism, based on the ability of Ea3 to absorb IL-3 activity, which does not involve the modulation of ATP. A second mechanism does not involve ligand–receptor interactions, based on the lack of absorption of IL-3 activity, which is associated with ATP concentrations. In the latter case the mechanisms by which IL-3 might function in the absence of receptors were not considered.

Recent studies have shown that when FDC-P1 cells are stimulated with IL-3 there is a rapid and transient redistribution of protein kinase C from the cytosol to the plasma membrane (Farrar *et al.*, 1985). A comparable effect was observed with phorbol myristate acetate. In identical studies (Farrar and Anderson, 1985) it was also demonstrated that IL-2 and PMA induced a redistribution of protein kinase C in an IL-2-dependent, cytotoxic T cell line. The significance of these observations and their possible relationship to regulation of growth are not known. Previous studies (Orosz *et al.*, 1983) have shown that PMA is directly mitogenic for IL-2-dependent T cell lines whereas PMA is not mitogenic for IL-3-dependent cells and in contrast inhibits IL-3-induced proliferation.

Protein kinase C is a Ca^{2+} and phospholipid-dependent enzyme which is activated by diacylglycerol (for a review see Nishizuka, 1984). In addition, protein kinase C has been shown to be the major receptor for and is activated by the tumor-promoting phorbol esters. Therefore in the above studies it was postulated that IL-3 may act through a phosphoinositide hydrolysis-dependent mechanism to generate diacylglycerol and cause the activation of protein kinase C. *In vitro* protein kinase C phosphorylates a wide variety of substrates primarily at seryl and threonyl residues but not at tyrosyl residues. One of the potential substrates has been shown to be the epidermal growth factor (EGF) receptor (Cochet *et al.*, 1984; Hunter *et al.*, 1984). Phosphorylation of the EGF receptor results in the reduction of EGF-stimulated tyrosine protein kinase activity. It is conceivable, therefore, that the PMA inhibition of IL-3 proliferation (Orosz *et al.*, 1983) is due to an effect of protein kinase C on the IL-3 receptor. Conversely, it is conceivable that IL-3 activation of protein kinase C represents a feedback mechanism for the regulation of the IL-3 receptor.

In fibroblasts, the regulation of cell growth by platelet-derived growth factor (PDGF) has been postulated to involve regulation of c-*myc* expression based on the rapid induction of c-*myc* transcription in quiescent fibroblasts

by PDGF (Kelly *et al.*, 1983) and by the ability of an introduced c-*myc* gene, under the regulation of a viral promoter, to partially abrogate the requirement for PDGF for growth (Armelin *et al.*, 1984). For these reasons c-*myc* expression has been examined in several IL-3-dependent cell lines (Kelly and Ihle, unpublished data). All the IL-3-dependent cell lines that have been examined express c-*myc* at levels which are comparable to those observed in fibroblasts. When cells are removed from IL-3, viability is rapidly lost as noted above and during the time periods that can be examined the levels of endogenous c-*myc* do not decrease appreciably. For this reason it has not been possible to directly determine whether IL-3 can induce c-*myc* expression.

To determine whether c-*myc* expression might be associated with IL-3-regulated growth, a series of recombinant murine retroviruses, capable of expressing the avian v-*myc* oncogene, were used to infect the IL-3-dependent FDC-P1 cells. For comparison, the effects on an IL-2-dependent cytotoxic T cell line were also examined (Rapp *et al.*, 1985). In both cases the expression of the v-*myc* oncogene was associated with the abrogation of a requirement for IL-3 or IL-2 for growth. In the case of the FDC-P1 line, the cells continued to express receptors for IL-3, however, they did not produce any detectable IL-3 or another mitogenic factor for the parental cell line and were not inhibited by an antiserum against IL-3. These results suggest that the abrogation of the requirement for IL-3 was not due to an autocrine type mechanism in which the cells produced their own growth factors.

With the recombinant viruses used it was possible to distinguish between the expression of the v-*myc* oncogene and the c-*myc* cellular gene by Northern blot analysis. The cells were therefore examined for the expression of c-*myc*. In neither the IL-2 nor the IL-3-independent cell lines was c-*myc* detectable and in neither case could growth factors induce the expression of the c-*myc* locus. These results were interpreted to indicate that the *myc* gene product may regulate either directly or indirectly its own transcription. This potential autoregulatory capacity had been previously postulated based on comparable effects in B cells (Leder *et al.*, 1983; Bernard *et al.*, 1983; Stanton *et al.*, 1983; Taub *et al.*, 1984; Rabbitts *et al.*, 1984). Nevertheless the results strongly suggest that the induction of expression of c-*myc* is an integral component of the pathways by which IL-3 or IL-2 regulate growth.

In addition to the *myc* oncogene, the *abl* oncogene, which is associated with Abelson MuLV, has been shown to abrogate the requirement for IL-3 for growth of IL-3-dependent mast cells (Pierce *et al.*, 1985). In these studies the effects of Ab-MuLV on the IL-3-associated differentiation and growth regulation of fetal liver cells *in vitro* were examined. From these cultures long-term cell lines were reproducibly isolated either from bulk cultures or from colonies. When the cells were characterized, they had the phenotypic and functional properties of mature mast cells; however, unlike normal IL-3-

induced mast cells, the transformed cells were immortalized for growth *in vitro* and did not require IL-3 for continued growth. As in the case of the v-*myc*-transformed cells, the Ab-MuLV-infected cells retained receptors for IL-3 and did not produce mitogenic factors which could stimulate the growth of normal IL-3-dependent mast cells. Therefore the abrogation of IL-3 dependence by v-*abl* is not due to an autocrine type mechanism and is due to the ability of the v-*abl* product to interupt the pathway of growth regulation by IL-3.

In the primary cultures of fetal liver cells in IL-3, Ab-MuLV gave rise only to mast cell lines and specifically did not give rise to myeloid lineage cells. These results suggest that Ab-MuLV does not affect the differentiation of IL-3-regulated cells and that cells which become committed to the myeloid pathway differentiate to mature granulocytes and macrophages. This contrasts with the apparent effects of v-*myb* or rearranged c-*myb* loci in which the effects noted are on the ability to differentiate and not on the growth factor dependence of the cells. Within this context it is also interesting to note that among the cells that IL-3 induces to proliferate *in vitro*, only mast cells retain the ability to proliferate for extended periods *in vitro*, as fully differentiated cells.

In recent studies we have also examined the effects of Ab-MuLV on IL-2-dependent growth. For these studies the IL-2-dependent helper T cell line (L2), described by Glasebrook and Fitch, was used. With infection of the cells with Ab-MuLV, independent cell lines were obtained (Ihle and Prystowsky, unpublished data). These results suggest the v-*abl* oncogene may also affect the growth pathway regulated by IL-2.

In recent studies the ability of Ab-MuLV to abrogate the IL-3 dependence of cell lines derived from retrovirus-induced tumors has also been examined (Oliff *et al.*, 1985). Similar to the studies of Pierce *et al.* (1985), Ab-MuLV was found to abrogate the requirements of the cells for IL-3. Whether the cells retained receptors for IL-3 was not assessed, although the data suggested that an autocrine type mechanism was not involved.

In contrast to v-*myc* and v-*abl*, a number of oncogenes affect the growth rate and immoratility of IL-3-dependent cells without affecting the IL-3 dependence of the cells. In particular Rein *et al.* (1985) examined the effects of Harvey sarcoma virus, which contains the Ha-*ras* oncogene, on cultures of spleen or fetal liver cells in the presence of IL-3. From these cultures long-term cell lines were readily obtained, indicating that the virus could immortalize the cells for growth *in vitro*. When the cell lines were characterized all were phenotypically and functionally mature mast cells, indicating that Ha-*ras*, like v-*abl*, does not affect the differentiation of the cells. In all the cases examined, the lines were polyclonal and expressed Ha-*ras* p21 at levels comparable to transformed fibroblasts. In spite of this, however, the cells still required IL-3 for growth. Similar results were obtained with cultures

infected with the Moloney sarcoma virus expressing the v-*mos* oncogene and the 3611 transforming virus, which expresses the v-*raf* oncogene.

Taken together the results suggest the regulation of growth by IL-3 may involve several components which in some cases may be altered by known oncogenes. The initial interaction envisioned is the binding of IL-3 with its receptor. Based on the results of other growth factor receptors it can be hypothesized that the IL-3 receptor is a protein kinase which is activated by binding of IL-3. With activation, the receptor can be envisioned to phosphorylate a cellular substrate which either directly or indirectly affects the transcription of the c-*myc* locus. In this context the v-*abl* protein kinase can be speculated to be able to phosphorylate the same substrates and, thereby, eliminate the requirement for IL-3. Consistent with this, c-*myc* expression is high in cells in which IL-3 dependence has been eliminated by infection with Ab-MuLV. The introduction of a v-*myc* oncogene, the transcription of which is under the regulation of a viral promoter, interrupts the signal transduction pathway at the terminal step.

The role of c-*myc* expression in the subsequent induction of DNA replication and cell division is not precisely known but has been shown not to be sufficient. In particular, in fibroblasts elegant studies (Stiles *et al.*, 1979; Pledger *et al.*, 1977; Smith and Stiles, 1981) have shown that PDGF only induces a competence in the cells and that the actual induction of proliferation requires additional factors. This phase of cell growth is termed progression and may involve other growth factors such as the insulin-like growth factors. This phase of cell growth therefore is independent of PDGF and its effect on c-*myc* expression. It can be speculated that it is this component of cell growth that is affected by transforming viruses such as the Harvey sarcoma virus.

In summary, most of the mechanisms involved in IL-3-regulated growth are not known. Of the aspects that are known there are a number of similarities between IL-3 and IL-2-regulated growth and, moreover, the growth regulation of fibroblasts by PDGF. These similarities would suggest that there is a common mechanism for growth regulation and that the only specificity conferred on various systems is through the receptors and the factors that they recognize. With regard to hematopoietic cells, the regulation of growth appears quite distinct from those genetic factors which regulate differentiation. Together, however, the factors which regulate differentiation, competence, and progression will have considerable significance in understanding the types of changes that are associated with malignant transformation of hematopoietic cells.

X. Summary and Conclusions

The mechanisms by which the immune system can influence the differentiation and proliferation of a variety of cell types are becoming better

understood. The major factors involved are a series of lymphokines which individually regulate the proliferation and differentiation of a number of lineages of cells. The past several years has resulted in the purification of a number of these factors to homogeneity and the cloning of cDNA and genomic clones for many of the factors. From these efforts the biochemical structure of lymphokines such as IL-2, IL-3, and GM-CSF has been deduced and the stage is set for the detailed analysis of the structure of these proteins as it relates to their biological activities. Considerable effort is now being directed at characterizing the receptors by which these factors mediate their effects. The progress which has been made with the IL-2 receptor over the past 2 years is striking. It is safe to assume that there also exists considerable interest in the characterization and cloning of the receptors for IL-3 and GM-CSF and it can be anticipated that we will know considerably more about the biochemical properties of the receptors in the near future.

To determine the mechanisms by which lymphokines such as IL-3, IL-2, or GM-CSF mediate their effects in differentiation and proliferation will involve considerably more effort. The first question deals with the mechanisms by which these factors induce proliferation of their target cell populations. What is the sequence of events by which IL-2, GM-CSF, or IL-3 induces a cell to enter cell division? As noted above, the possibility exists that lymphokines may function in a manner very much like other growth factors such as PDGF or EGF which will help to unravel the mechanisms involved. The integral relationship of various cellular homologs of oncogenes to growth regulation has provided an extraordinary means to identify genes which are involved in the regulation of cell proliferation. Because of the stringent requirements of hematopoietic/lymphoid cells for the growth factors which have been characterized, these systems are quite amenable to research directed at growth regulation. As information evolves it will be of considerable interest to assess whether a common pathway of growth regulation is involved in which specificity is conferred only by the growth factor specificity of the cell receptors.

The second question is much more characteristic of hematopoietic systems and early lymphoid lineages and deals with the mechanisms involved in differentiation. The dynamic characteristics of this response are most evident in the response of normal bone marrow cells to IL-3. In this response there is an obligatory sequence of changes in gene expression which occurs and results in differentiation. Associated with these changes are probability factors which determine the frequency with which cells become committed to specific patterns of changes in gene expression. A central question is what types of genes can control these sequential changes. Are there factors, including growth factors such as IL-3, which can alter the patterns of differentiation or is differentiation controlled totally by internal, genetically defined events? The availability of hematopoietic growth factors and *in vitro*

systems which support differentiation under defined conditions has provided a means to begin to more precisely define the sequences of differentiation. This, in turn, will provide the systems necessary to begin to identify genes that may be involved in these functions. As with growth regulation, viral oncogenes and their normal counterparts may provide the first insights into the types of genes which may be involved. In this context, the possible role of c-*myb* is of particular note and may be the first oncogene to be shown to specifically affect differentiation without affecting growth regulation. Ultimately the significance of the above events must be put within the context of normal and pathological functioning of the hematopoietic/lymphoid systems. As emphasized in this review, evidence exists to suggest that the regulation of hematopoietic stem cell function involves a constitutive system and an immunologically regulated system. Evolutionarily it can be envisioned that hematopoietic regulation evolved well before the immune system. Within the evolution of the immune system it can be speculated that there were selective advantages for the ability to increase hematopoietic cell types during immune responses. Irrespective, it is unclear how these systems, through their respective growth factors, are related. For example, does IL-3 simply increase the rate of proliferation of progenitors or does IL-3 also increase the number of potential lineages. The limiting concentrations of the immune factors *in vivo* relative to the constitutive factors support the regulatory capacity of this group of factors. It will, therefore, be important to specifically assess the effects of activation of helper T cells *in vivo* within the context of hematopoietic/lymphoid stem cell function. These types of studies will also be of importance as sufficient quantities of growth factors are available for *in vivo* experiments.

The demonstration that cyclosporin A can specifically affect the production of helper T cell-derived growth factors will be important in a variety of ways. Past research has emphasized the effects of this drug in situations in which substantial immunological suppression is required. However, the question exists whether there are situations in which more subtle effects on the production of lymphokines may be of importance. In this regard, the unique requirement of mast cells for IL-3 for maintenance of viability might suggest that cyclosporin A would be useful in allergic situations. Other diseases which are characterized by chronic inflammation, for example, may also be of interest to examine. Finally, as noted above, certain types of leukemias are characterized by requiring T cell-derived growth factors at certain stages. This opens the possibility that cyclosporin A may be therapeutic in these situations. Nevertheless, cyclosporin A should prove to be of considerable interest in the future for experimental purposes as well as perhaps providing unique therapeutic approaches.

Our interests have been with the potential role of the immune responses in leukemogenesis. In particular we have focused on how altered immuno-

logical regulation may increase the risk for leukemia and how transformation affects the regulation and differentiation of hematopoietic/lymphoid cells. Within the context of myelogenous leukemias, considerable evidence now exists to implicate T cell-derived growth factors in several aspects. The chronic production of growth factors in response to antigenic challenge increases the number of cells at risk for transformation. In the systems we have explored, this has involved retroviruses although it can be speculated that other antigens, when present at high enough concentrations for long enough periods of time, should have comparable effects. Among the transforming events which occur some affect differentiation and some affect growth regulation. Among those affecting growth regulation, certain types of transformation affect growth rate without affecting growth factor dependence whereas other types of transformation specifically involve the abrogation of growth factor dependence. Between these functions, we can begin to appreciate the significance of the progression of myeloid leukemias and equate certain phenotypes with the effects seen with certain oncogenes. These types of effects can be anticipated to occur in other lineages.

In this review we have attempted to summarize the literature concerned with T cell-derived lymphokines which regulate hematopoietic/lymphoid lineages. The paucity of data in many areas has offered the opportunity to speculate on a variety of topics. Our intent in speculating is not so much to provide a statement of our position but rather to provoke thought. Hopefully we have succeeded.

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ADVANCES IN IMMUNOLOGY, VOL. 39

Antigen Presentation by B Cells and Its Significance in T–B Interactions

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I. Introduction

Interest in the question of whether B cells can present antigen to T cells emanates from a consideration of two findings in relation to the immune response to T-dependent antigens. The first observation, made in the late 1960s and early 1970s by Mitchison (1, 2) and by Rajewsky *et al.* (3), showed that the antigenic determinants recognized by T cells and B cells must be present on the same macromolecule in order for effective antigen-specific T cell help to be delivered to B cells. The second important discovery to be considered for T-dependent responses was that the accessory cells had to "process" the antigen and display it in the context of the MHC-encoded Ia molecules for the recognition by and activation of helper T cells (4–8). Previous to these findings the phenomenon of hapten–carrier-linked recognition of antigen by T and B cells had been interpreted as strong evidence in support of the concept that in order to obtain effective T–B cooperation, antigen had to serve as a bridging unit to allow the physical interaction between carrier-specific T cells and hapten-specific B cells and that this interaction was required for an effective immune response (1, 2). However, since in order for T cells to recognize and respond to antigen, the antigen must be processed and presented by an accessory cell in the context of MHC gene products, the postulate that antigen forms a simple bridge between a carrier-specific T cell receptor and a hapten-specific B cell receptor became untenable. This apparent discrepancy between the concept of antigen bridging for effective T–B interactions and the requirement of accessory cell processing and presentation of antigen for T cell recognition would be reconciled if B cells could serve as antigen-presenting accessory cells. That is, if B cells could process antigen and present it in the context of the B cell Ia molecules, antigen-specific T cells would be specifically focused by the MHC/processed antigen onto the appropriate antigen-specific B cells thus initiating the cellular interactions involved in mounting a T-dependent immune response (9). The purpose of this article is to review the evidence in favor of the concept that B cells can in fact serve as antigen-presenting accessory cells to T cells of the helper/inducer subset as well as the experi-

ments that suggest that this is a critical step in the differentiation of B cells for a T-dependent immune response.

II. The Capacity of B Cells to Serve as Antigen-Presenting Cells

A. Antigen Presentation by Normal B Cells

At about the same time in the late 1970s several groups of investigators initiated studies to test the ability of B cells to present antigen to T cells. During this time, Bergholtz and Thorsby (10) studied the ability of HLA-DR expressing human B lymphocytes to substitute for macrophages in presenting PPD or the hapten, trinitrophenyl (TNP) (in the form of TNP-autologous B cells) to human T cells. Their observations suggested that whereas B cells were unable to present the soluble antigen purified protein derivative (PPD), TNP-modified B cells could stimulate the proliferation of T cells that had been initially primed with TNP-monocytes. Hiramine and Hojo (11) examined the ability of guinea pig B cells to present PPD or testicular antigen to purified lymph node T cells from PPD or testicular antigen-primed T cells. Their findings, in contrast to those of Bergholtz and Thorsby, revealed that B cells were able to present both these antigens to T cells, as measured by T cell proliferation, but they were approximately one-half to one-third as effective as macrophages. Kammer and Unanue (12), using mice, investigated the ability of macrophage-depleted spleen cells to present keyhole limpet hemocyanin (KLH) to KLH-primed T cells. Their results showed that an Ig^+ population was able to present KLH resulting in T cell proliferation, but on a per cell basis the Ig^+ cells were approximately 100-fold less efficient than macrophages. Although in this latter study considerable care was taken to eliminate contaminating non-B antigen-presenting cells from both the B and T cell populations, it remained difficult in any of the above studies to eliminate the possibility that sufficient contaminating macrophages or dendritic cells were present to provide the observed antigen-presenting function.

The efforts of our laboratory to test the hypothesis that B cells could present antigen focused on the unique ability of B cells to bind antigen via their surface Ig, and began by utilizing rabbit anti-mouse Ig (RAMIG) antibodies as the antigen (13). RAMIG had two important advantages over other conventional antigens for assaying the ability of B cells to present antigen. First, RAMIG binds polyclonally to B cells irrespective of the antigen specificity of their receptor (in contrast to the very low frequency of antigen-specific B cells which, for any given antigen, would make detection of antigen presentation very difficult). Second, normal rabbit γ-globulin (NRGG),

which would be used to prime the T cells *in vivo* and which should be presented as effectively as RAMIG by macrophages, would not bind to B cell Ig and thereby could serve as a control antigen to detect macrophage contamination of the B and T cell preparations.

B cells, prepared from the spleens of normal mice, were cultured with T cells isolated from the lymph nodes of mice primed with NRGG in the presence of antigen, either RAMIG or NRGG. The results we observed, shown in Table I, demonstrated that antigen bound via mIg could be presented to T cells as measured by the induction of a T cell proliferative response. Specifically, using spleen adherent cells as a source of macrophages and dendritic cells we found that both NRGG and RAMIG were presented with similar efficiency. As mentioned above, this was expected since RAMIG should not be bound or processed any differently than NRGG by macrophages. However, when B cells were used as the antigen-presenting cells, RAMIG was presented as effectively by the B cells as it was by macrophages, while NRGG, which could not bind to the surface Ig on B cells, was not presented. As described above, this latter finding demonstrated that it was indeed the B cells which were the antigen-presenting cells in this system and not the result of contaminating macrophages.

The antigen specificity of the response was demonstrated by the observation that B cells cultured in the presence of RAMIG did not cause the proliferation of T cells primed to an unrelated antigen, ovalbumin. Additional studies demonstrated that the response of NRGG-primed T cells to RAMIG presented by B cells was MHC restricted, and that the $F(ab')_2$ fragments of RAMIG worked as well as intact IgG. Also, further controls were carried out to ensure that the antigen presentation observed could not

TABLE I

PRESENTATION OF RABBIT ANTI-MOUSE IMMUNOGLOBULIN TO RABBIT IgG-PRIMED T CELLS BY MACROPHAGES AND B CELLS

Antigen-presenting cells	Antigen	[^{3}H]Thymidine incorporation[a] (E − C; cpm × 10^{-3}) in experiment number			
		1	2	3	4
Macrophages	NRGG	124	234	121	142
Macrophages	RAMIG	99	186	103	144
B cells	NRGG	0	0	4	0.5
B cells	RAMIG	169	68	148	32

[a] [^{3}H]Thymidine incorporation expressed as experimental (E) cpm minus unstimulated control (C) cpm.

be due to contaminating macrophages or dendritic cells. Taken together, the data strongly suggested that B cells could take up antigen via their mIg and present the antigen to T cells in a manner analogous to macrophages.

B. Antigen Presentation by B Cell Lymphomas

Although substantial efforts had been made to ensure that contaminating, non-B antigen-presenting cells had been eliminated in the experiments designed to examine the capacity of normal B cells to present antigen, it was important to reproduce these findings using *in vitro* cloned sources of B cells and T cells so that any possibility of other cells contributing to the response could be eliminated. McKean and collaborators (14) investigated the capacity of several *in vitro*-grown, Ia^d-bearing B cell tumors to present antigen to an *in vitro*-propagated, Ia-restricted, antigen-specific T cell line. Using the synthetic terpolymer, GAT, as antigen these workers found that the B cell lymphomas were able to present GAT to the T cell line resulting in a 4- to 6-fold increase in T cell proliferation. Glimcher and co-workers (15) conducted similar experiments showing that Ia-bearing B lymphoma lines could present the soluble protein antigens ovalbumin, PPD or the synthetic terpolymer GLϕ to either primed T cells or propagated T lymphoblasts specific for the various antigens.

The difficulty with these experiments was that the maintenance of T cell lines required the frequent restimulation of the T cells with spleen accessory cells plus antigen. Therefore, the possible contamination of T cells with autologous accessory cells could not be ruled out. In an effort to circumvent this problem a series of experiments were conducted in which cloned *in vitro*-grown B cell lymphoma lines, originally isolated, characterized, and shown to express Ia molecules by Kim *et al.* (16), were tested for their ability to present antigen to cloned antigen-specific, Ia-restricted T cell hybridomas which were also propagated *in vitro* (17) and by virtue of their tumor properties required no antigen-presenting cells or antigen for their continued growth. The initial experiment was conducted using the B cell lymphoma, BAL 17.7.2, which expresses $I\text{-}A^d$ and $I\text{-}E^d$. BAL 17.7.2 cells were tested for their ability to present the antigen KLH to the KLH-specific $I\text{-}E^d$-restricted T cell hybridoma, AODK1.16, as detected by IL-2 production (18). The results clearly demonstrated the capacity of BAL 17.7.2 cells to present KLH to the T cell hybridoma and, thus, proved conclusively that B cells, albeit a B cell tumor, could present antigen to T cells, i.e., a T cell hybridoma, in the absence of contaminating non-B accessory cells.

Similar studies, by Walker *et al.* (19), using T cell hybridomas with different antigen specificities and MHC restrictions, demonstrated that all 14 of the B cell tumors tested which expressed Ia molecules were able to present antigen to the T cell hybridomas.

C. Comparison of Ig- and Non-Ig-Mediated Uptake of Antigen by B Cells

The studies mentioned above clearly indicate that B cells can take up antigen either by their Ig receptors (in the case of RAMIG) or by other non-Ig-mediated means (in the case of B lymphomas) and that both pathways can lead to the appropriate presentation of antigen to T cells. In the context of the proposed role of B cell antigen presentation under physiological conditions, the capacity of B cells to present antigens which do not bind to their Ig could result in the undesirable secretion of antibodies specific for a large variety of antigens unrelated to the antigen being presented. However, because of the relatively large amounts of antigen required to stimulate T cells when B cells not specific for the antigen are used as antigen-presenting cells (APC), together with the likelihood that antigen-specific B cells would be far more efficient than other B cells in taking up antigen, it was thought that there might be considerable quantitative differences in the B cell's ability to present antigen depending upon the means of antigen uptake. To test this, the RGG-primed T cell system was utilized and a comparison was made between the capacity of the $F(ab')_2$ of normal rabbit IgG (NRGG) and that of the $F(ab')_2$ of rabbit anti-mouse Ig (RAMIG) to stimulate RGG-primed T cells when activated B cells were used as a source of antigen-presenting cells (20). The results of this study are illustrated in Fig. 1. When NRGG $F(ab')_2$-pulsed B cells were used to stimulate NRGG-primed T cells, it was necessary to pulse the B cells with 2–6 mg of antigen in order to obtain any significant T cell proliferation. In contrast, when RAMIG $F(ab')_2$ was used less than 1 μg was needed to stimulate a similar proliferative response. Since the RAMIG preparation used in this experiment was only 8% specific antibody, these data indicate that RAMIG $F(ab')_2$, taken up by the surface Ig receptors, was at least 10^4-fold more efficient an antigen than the NRGG $F(ab')_2$ that was taken up by the B cells by a non-Ig-mediated means. In contrast to these findings, when macrophages were used as a source of accessory cells NRGG and RAMIG had similar efficiency with respect to their capacity to serve as antigen for RGG-primed T cells (see Table I).

More recent studies have confirmed and extended these findings. In a study by Tony and Parker (21), the RAMIG and NRGG comparison was extended with the use of RGG-specific T cell clones and T cell hybrids and the capacity of specifically purified rabbit anti-μ or anti-δ antibodies to stimulate these T cell clones and hybrids was measured. Similar to the findings with polyclonal RGG-primed T cells, these workers found a 10^4-fold difference between the capacity of normal rabbit γ-globulin and specific antibody to surface immunoglobulin to stimulate a T cell proliferative response or IL-2 production. Furthermore, they were also able to study the effect of

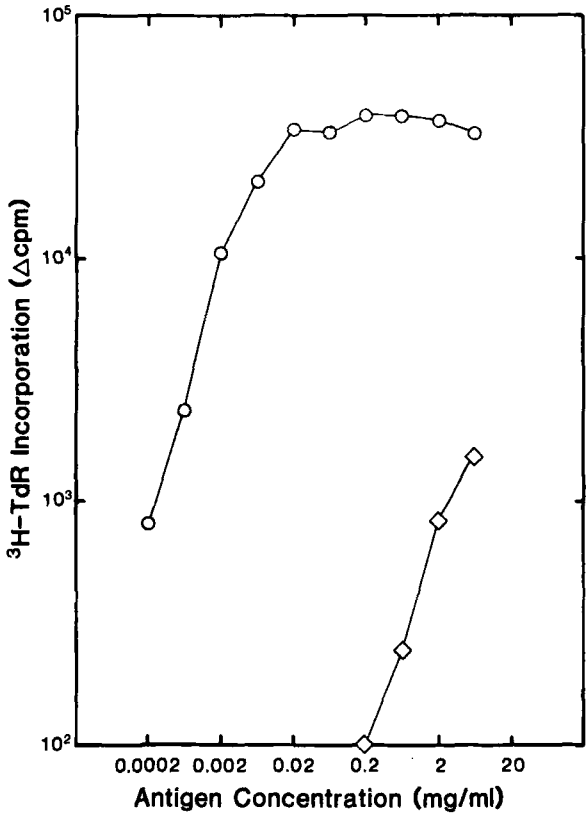

FIG. 1. Comparison of RAMIG-F(ab')$_2$ to NRGG-F(ab')$_2$ with respect to their efficiency in stimulating proliferation by NRGG-primed T cells when LPS-activated B cells were used as antigen-presenting cells. B cells activated with LPS for 24 hours were washed, irradiated (4000 R), and pulsed with different concentrations of RAMIG-F(ab')$_2$ (○) or NRGG-F(ab')$_2$ (◇) before being placed in culture with 4×10^5 NRGG-primed T cells.

this antigen presentation by B cells on the subsequent proliferation and differentiation of the B cells. Their studies demonstrated that not only did B cells present antigen and stimulate the T cell hybrids and T cell lines to elaborate IL-2, but also this interaction led to B cell activation as measured by B cell proliferation and Ig secretion. These findings represent important further evidence of the potential role of antigen processing and presentation by B cells in the T–B collaboration required for an antibody response.

A somewhat different approach has been taken by Abbas and co-workers (22, 23) and Lanzavecchia (24). In the studies of Abbas and Rock, hapten

(TNP)-specific B cells were purified from hapten-primed or normal animals and these cells were studied for their capacity to present hapten-conjugated carrier or unconjugated carrier to carrier-specific T cell hybrids. It was found that TNP-specific B cells were capable of stimulating IL-2 production by GLϕ-specific T cell hybrids with very small amounts of TNP–GLϕ (10–100 ng) whereas approximately 1000-fold more unconjugated GLϕ was required to achieve a similar degree of stimulation of these T cell hybrids when the same TNP-specific B cells were used as antigen-presenting cells. This low dose antigen presentation by the hapten-specific B cells showed hapten specificity in that an unrelated hapten conjugated to GLϕ was only stimulatory at the same high dose of antigen required for stimulation when the unconjugated carrier was used.

The use of antigen-specific B cells as APC has also been described recently with human cells by Lanzavecchia who utilized Epstein–Barr virus (EBV)-transformed human B cell lines specific for tetanus toxoid and T cell clones specific for the same antigen. As shown in Fig. 2 his study indicated that extremely small amounts of antigen (10^{-11}–10^{-12} *M*) were capable of stim-

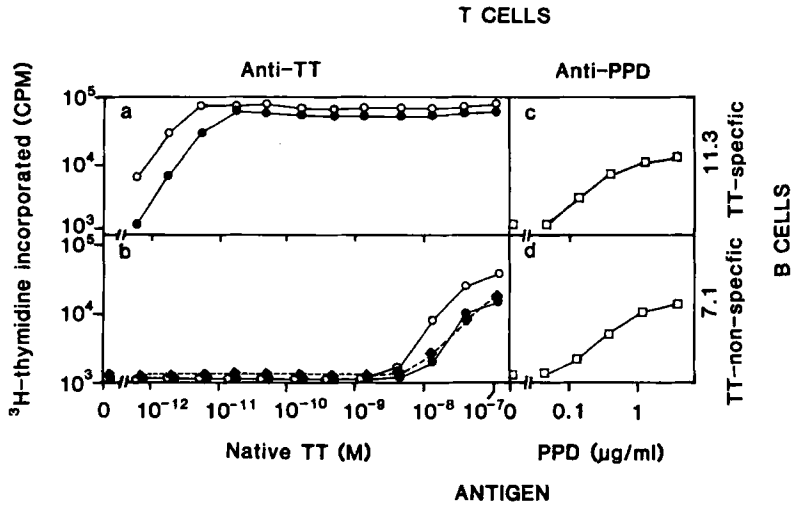

FIG. 2. Antigen presentation by antigen-specific B lymphocytes. Cells from TT-specific T cell clones KT2 (●) and KT4 (○) or from a PPD-specific T cell line (□) were cultured with an irradiated EBV-transformed B cell clone specific for TT (clone 11.3, a, c) or a non-TT-specific clone (7.1, b, d). The cultures were stimulated with a wide range of concentrations of native TT or PPD. In b, clone KT2 was also stimulated in the presence (◆) of 3 μg/ml purified antibody produced by clone 11.3. After 2 days, the cultures were pulsed with [^{3}H]thymidine.

ulating T cell proliferation when tetanus toxoid (TT)-specific EBV-transformed B cell lines were used. When EBV-transformed cells without specificity for tetanus toxoid were used, as in the previously mentioned studies, approximately 10^4-fold higher antigen concentration was required to achieve a comparable degree of stimulation. Furthermore, when TT-specific B cells were compared with B cells lacking specificity for TT for their ability to present another antigen, PPD, no difference between the two lines was observed. T cell stimulation was shown to be MHC-restricted with respect to HLA-DR compatibility and was inhibited by anti-HLA-DR antibodies. The requirement for processing of the tetanus toxoid by the antigen-specific EBV-transformed B cells was demonstrated by fixation and chloroquine treatment experiments similar to those described in the murine system (see Section II,B). Lanzavecchia (personal communication) was also able to examine the effect of this antigen presentation by B cells on the subsequent differentiation of the EBV-transformed B cells. Using the same low antigen concentration required for T cell proliferation (10^{-11} to 10^{-12} *M*), Ig secretion by the EBV-transformed B cells was increased and the spontaneous proliferation of an EBV-transformed B cell line was decreased. Thus, antigen presentation by these B cell lines resulted in T cell stimulation which in turn led to terminal differentiation of the B cell line as evidenced by increased immunoglobulin production and decreased cellular proliferation. A detailed analysis of the amount of antigen required for stimulating T cells together with measurements of the affinity of the antibody present on the B cell surface has led Lanzavecchia to conclude that a very low degree of Ig occupancy occurred during antigen uptake by B cell immunoglobulin. At 10^{-11} to 10^{-12} *M* concentration of antigen less than 1% of the immunoglobulin receptors would be expected to be occupied at any one time. It is likely that this very low degree of binding ($<10^3$ molecules/B cell) might be insufficient to allow a critical quantity of antigen to be processed and presented, so that repetitive cycles of binding and endocytosis over a prolonged period of time might be necessary for sufficient amounts of processed antigen to accumulate at the cell surface before T cell triggering could be initiated. Clearly further studies on the kinetics of processing and the turnover of processed antigen must be performed to obtain a greater insight into this matter; however, the calculations and postulation made by Lanzavecchia are quite suggestive that a continuous uptake, processing and accumulation of antigen might be an integral part of the antigen presentation mechanism by B cells.

The work described in this section has unequivocally demonstrated the ability of both normal B cells and B cell tumors to present antigens to T cells and that the antigen can be taken up by the B cell via both membrane Ig and by non-Ig-mediated mechanisms. While we can currently only speculate on the importance of these two mechanisms *in vivo*, from the published results

comparing Ig and non-Ig-mediated uptake of antigen, it is clear that antigen taken up via immunoglobulin receptors is several orders of magnitude more efficient, with respect to the concentration of antigen required to effect a given amount of T cell stimulation, than antigen taken up by non-Ig-mediated means. This observation is probably crucial with respect to the physiologic significance of antigen presentation by B cells. The amount of antigen that a B cell would be exposed to under *in vivo* physiologic circumstances would rarely, if ever, achieve the concentrations required for B cells to be effective antigen presenting cells when uptake only occurs by non-Ig-mediated means (100–1000 μg/ml). Thus, with lower antigen concentrations, only antigen-specific B cells would take up, process, and display a sufficient quantity of antigen to result in meaningful interactions with an antigen-primed T cell. In fact, when animals are experimentally exposed to the very high concentrations of antigen required for such "bystander" B cell presentation of antigen, immunologic unresponsiveness is often observed (25, 26). It is possible that in such "high zone" tolerance, the capacity of antigen nonspecific B cells to present antigen and focus carrier-specific T cells to them thereby effectively deviating the usual interactions between antigen-specific T and B cells may be one of the mechanisms involved in the induction of this type of unresponsiveness.

III. The Fate of Antigen Taken up by B Cells

A. Biochemical Studies

Since the macrophage has for several years been considered the prototype of an antigen-presenting cell it is useful to compare B cells and macrophages with respect to how antigen is handled by these two types of accessory cells. As is detailed below, rather striking quantitative and, in some instances, qualitative differences were shown to exist between these two cell types when the biochemical fate of radiolabeled proteins was examined. For these studies radioiodinated proteins were used and the uptake and fate of these proteins was evaluated by studying the generation of proteolytic breakdown products, as measured either by polyacrylamide gel electrophoresis or the generation of acid-soluble radioactivity. Also, the effect of certain inhibitors of cellular functions on the degradation of the radiolabeled proteins was examined.

In order for an accessory cell to present antigen to T cells it must first take up that antigen. There are three mechanisms by which antigen uptake can be accomplished. The first involves fluid phase pinocytosis of the antigen without significant binding to the cell surface prior to endocytosis. The second and third mechanisms both involve the binding of antigen molecules

to the cell surface but differ in that one mechanism involves antigen binding via specific receptors (e.g., Ig receptors for antigen, Fc receptors, C3 receptors, lectins) located on the accessory cell surface while the other is mediated via non-receptor-binding of macromolecules to the accessory cell surface. Most protein antigens are taken up by this latter "nonspecific" mechanism. However, with the definition of more receptor-like molecules on the surface of accessory cells it is possible that in the future our concept of the specificity of binding of proteins to the cell surface will change.

When fluid phase pinocytic capacity was compared between normal B cells, B cell lymphomas, and macrophages, striking differences were observed when uptake of horseradish peroxidase was used as an indicator of fluid phase pinocytic activity (18). Normal macrophages and a macrophage-like cell line (P388D1) had a pinocytic rate of approximately 0.5–1.0 $\mu l/10^7$ cells/hour. In contrast to this, B cell tumors, although of approximately the same size as the macrophages, were much less active, pinocytosing fluid at a rate of approximately 0.01 $\mu l/10^7$ cells/hour. Normal B cells were even less active than B cell tumors with a pinocytic rate of approximately one-tenth that of the B cell tumors. Similarly, when the adsorption of iodinated protein to the cell surface of B cells and macrophages was compared, striking differences were observed (18). Using the protein ^{125}I-labeled KLH to measure non-receptor-mediated binding to the surface of these cells, under conditions which inhibited endocytosis, macrophages were shown to bind approximately 17 ng of protein/10^7 cells, B cell tumors approximately 2 ng/10^7 cells, and normal B cells 0.3 ng/10^7 cells. The extent of binding was dependent upon the quantity of protein incubated with the cells and, as had been previously demonstrated for uptake of antigen by macrophages (27, 28), was not a readily saturable process so that the amount of binding increased progressively with the amount of protein added to the cells. The relatively low uptake of antigen by B cells compared to that of macrophages, when the antigen used was taken up by either fluid phase pinocytosis or by non-receptor-mediated adsorption to the cell surface, stood in striking contrast to the results obtained when a protein capable of binding specifically to the immunoglobulin receptors on B cells was studied. That is, when radiolabeled rabbit anti-mouse immunoglobulin (RAMIG) was used as the protein, compared to macrophages, B cells took up approximately equivalent amounts of this protein by virtue of the capacity of RAMIG to bind to the approximately 10^5 molecules/cell of surface Ig (29). This is undoubtedly of crucial importance in allowing antigen-specific B cells to specifically take up sufficient amounts of antigen via its membrane Ig to allow presentation to T cells especially at levels of antigen that would be too low to allow for non-antigen-specific B cells to take up sufficient antigen to allow effective presentation to T cells. This aspect has been explored in detail in Section II,C.

If, as is elaborated below, antigen presentation by accessory cells requires

an active processing event which involves the proteolytic degradation of soluble protein antigens into peptides, then the characterization of the capacity of B cells to endocytose and degrade antigens would be an important function of these cells. Thus far there have been no studies of the adsorptive endocytic capacity of B cells compared to macrophages by ultrastructural analysis. However, some information is available using the technique of proteolytic stripping of surface bound protein. This procedure gives semiquantitative information with respect to the efficiency of endocytosis of adsorbed protein by measuring that portion of the cell-associated protein that is protected from the effects of proteolysis presumably due to endocytosis and sequestration of the protein in an intracellular compartment (30, 31). Using this method, after a 2-hour incubation at 37°C, both macrophages and B cells could be shown to internalize approximately 30% of rabbit anti-mouse immunoglobulin bound to their surface. However, antigen (KLH) bound nonspecifically to the surface of B cells was not detectably compartmentalized into a protease-resistant site (29). In contrast, when KLH was bound to the surface of peritoneal macrophages approximately 20% of the protein was internalized during the same time interval. Thus, the extent of sequestration of protein into protease-resistant sites depends upon the mode of binding to the B cell surface: rabbit anti-mouse Ig interaction with cell surface Ig leads to efficient internalization whereas antigen binding to nonspecific membrane sites does not.

The relative capacity of B cells and macrophages to degrade proteins initially bound to their cell surface was studied by measuring the release of acid soluble radioactivity during a period of incubation at 37°C (Table II) (29). It is clear from the data in this table that antigens bound to the surface of B cells via non-Ig-mediated mechanisms were very inefficiently degraded compared with the fate of the same antigen taken up by macrophages. The rate of protein degradation as measured by the generation of TCA-soluble radioactivity was 1–5% of the rate observed in macrophages. In striking contrast to the data obtained with proteins taken up nonspecifically by B cells, when the degradation of RAMIG was studied, B cells and macrophages displayed comparably high rates of degradation (29, 32, 33). Thus, it appears that ligand bound to membrane immunoglobulin is preferentially delivered to a site of catabolism where it is degraded at a rate comparable to that of macrophages. Antibodies to other surface antigens such as β_2-microglobulin were degraded at much slower rates, similar to proteins taken up nonspecifically as were immune complexes bound to B cell Fc receptors. This striking difference in catabolism between Ig-mediated and other mechanisms of uptake of antigen by B cells may be another critical factor in determining their preferential capacity to function as efficient APC for T cells that are specific for the same antigen as the B cells.

In summary, these data suggest that although B cells have the machinery

TABLE II
DEGRADATION OF PROTEINS TAKEN UP BY NORMAL B CELLS, B CELL LYMPHOMAS (BAL), AND MACROPHAGES (PEC)

Experiment	Cell	^{125}I-Labeled antigen	^{125}I-Labeled protein bound (cpm × 10^{-3})	Degraded/hour (%)
1	PEC	KLH	449	40.1
	BAL	KLH	13	<0.1
2	PEC	D-OVA	112	18.6
	BAL	D-OVA	33	1.1
3	PEC	RAMIG	294	13.4
	BAL	RAMIG	136	7.0
	n1B	RAMIG	137	6.5
4	PEC	KLH-anti-KLH	636	8.9
	BAL	KLH-anti-KLH	268	0.1
5	PEC	RAMIG	283	14.4
	BAL	RAMIG	219	8.7
	PEC	Anti-β_2m	39	11.9
	BAL	Anti-β_2m	39	0.1

(presumably lysosomes) to catabolize protein with an efficiency similar to that of macrophages, the manner in which the antigen is initially bound to the cell surface appears to dictate whether or not it is efficiently endocytosed and catabolized. Nonreceptor-mediated binding, binding to Fc receptors, and binding to class I H-2 molecules do not lead to efficient delivery of the protein to the site of catabolism, whereas binding to surface Ig does.

B. FUNCTIONAL STUDIES

Earlier studies utilizing macrophages as accessory cells strongly suggested that active metabolic events were required following the binding of antigen to the accessory cell surface, before antigen could be effectively presented to and recognized by T cells. The evidence used to support the active "processing" of antigen included (1) a finite time of incubation at 37°C after antigen was bound to the cell surface had to elapse before macrophages could present antigen effectively to T cells (34, 35); (2) inhibitors of energy requiring metabolic events prevented antigen presentation to T cells (36); (3) antigen became resistant to antibody blockade (30, 37) or protease stripping (30, 31) of the cell surface after incubation of antigen pulsed macrophages at 37°C for several hours; (4) lysosomotropic amines inhibited antigen presentation by macrophages (38, 39); and (5) inactivation of macrophages by ultraviolet light or aldehyde fixation before exposure to antigen prevented effective antigen presentation even in the presence of added interleukin 1 (IL-1) (35, 39, 40).

Such studies suggested that internalization and limited proteolysis of antigen may be necessary events that must occur prior to presentation of antigen by macrophages. Since, as detailed above in Section III,A, there are striking differences in the efficiency with which B cells and macrophages bind, endocytose, and degrade antigen, the possibility existed that the requirements for processing of antigen might differ depending upon the accessory cell involved. To examine this, macrophages and B cell lymphomas were tested for their capacity to present antigen taken up by nonspecific means to antigen-specific T cell hybrids as measured by the production of IL-2 by the hybrids following interaction with antigen pulsed accessory cells. To study the kinetics of antigen presentation, antigen was allowed to bind in the cold to the surface of the accessory cells followed by washing and incubation at 37°C for varying periods of time, after which the cells were mildly fixed with paraformaldehyde. Both B cell lymphomas and macrophages exhibited similar kinetics in that no antigen-presenting capacity was demonstrable prior to 45 minutes at 37°C at which time some minimal activity was observed with B cell tumors which increased over a period of about another hour. Similarly, peritoneal exudate macrophages started to show antigen-presenting capabilities at 60 minutes which progressively increased for the next hour.

Several groups (37, 41–44) have shown that there is a high degree of cross-reactivity between native and denatured forms of the same protein when T cell recognition is studied but this is not observed at the antibody or B cell level. It has been suggested (45) that the reason for this is that both native and denatured antigens are processed by accessory cells so that common determinants not involving three-dimensional configuration are generated as a result of antigen degradation. Thus, the pattern of cross-reactivity between native and denatured antigen might be useful as a marker for antigen processing. With this line of reasoning in mind, studies were performed to determine whether B cells were also capable of presenting antigen in such a way as to render native and denatured proteins cross-reactive at the T cell level (39). For this purpose B cell lymphomas or macrophages were used to present native or denatured ovalbumin to an ovalbumin-specific T cell hybridoma. It was observed that this hybridoma reacted equally well to either native or denatured ovalbumin over a wide range of antigen concentrations when either B cells or macrophages were used as APC. Thus, B cells have the same ability as macrophages to present native and denatured protein antigen in a manner that is recognized as cross-reactive by T cells.

Somewhat stronger evidence for proteolytic degradation as an important processing event has come from the study of the effect of a variety of weak bases, including chloroquine, on the antigen-presenting capacity of accessory cells. These "lysosomotropic" agents have been shown to be concentrated within lysosomes, raise the lysosomal pH, and, as a consequence, inhibit the

function of a variety of acid hydrolases contained within this organelle (46). When chloroquine was added prior to and during the time required for antigen processing it was found that both B cell lymphomas and peritoneal exudate macrophages were similarly inhibited in their capacity to present antigen to T cell hybrids in a dose-dependent fashion with concentrations ≥ 40 μM being completely inhibitory (39). In other experiments this inhibition was demonstrated to be restricted to the processing events rather than having an affect on antigen display, since cells that were pulsed with antigen prior to chloroquine treatment showed a normal capacity to present that antigen despite subsequent treatment with the lysosomotropic agent prior to testing for antigen-presenting capability. Furthermore, as alluded to in Section II,C, lysosomotropic agents and aldehyde fixation have been shown to have similar effects on the capacity of human antigen-specific B cells to process and present antigen (24). In these studies it was shown that pretreatment of tetanus toxoid-specific EBV-transformed B cells with chloroquine or glutaraldehyde inhibited their capacity to present tetanus toxoid to tetanus toxoid-specific T cell lines in a manner completely analogous to that which was shown for B cell tumors that take up antigen by nonspecific means. Thus, both Ig-mediated and non-Ig-mediated uptake of antigen by B cells appear to go through operationally similar processing events.

With several of the T cell hybridomas that were used for the studies described above, it has been possible to define the processing event more definitively as involving proteolytic degradation. The results of this work demonstrated that accessory cells, either macrophages or B cells, that were fixed with paraformaldehyde or glutaraldehyde prior to exposure to antigen, while they were totally incapable of presenting intact protein antigen to the T cell hybridomas, were very efficient in presenting certain peptides derived from that antigen that had been generated by either enzymatic or chemical cleavage of the antigen prior to its being added to the fixed presenting cells (47, 48). One example of this type of experiment is shown in Table III which demonstrates that both peritoneal exudate macrophages and B cell lymphomas which have been prefixed before addition to the culture have a similar capacity to present a tryptic or cyanogen bromide (CNBr) peptide of ovalbumin to the 3DO54.8 hybridoma, whereas both cell types when prefixed failed to present the intact native or denatured antigen. Although the studies shown in this table were performed with a B cell lymphoma, identical results have been obtained with normal mitogen-activated B cells. The differences between mitogen-activated and resting B cells in their antigen processing and presenting capabilities are discussed in Section IV.

Finally, it is of some interest that in recent studies comparing different antigens we have found considerable heterogeneity in terms of the time required for processing to take place. Thus with KLH and ovalbumin, anti-

TABLE III
CAPACITY OF PREFIXED B CELLS AND MACROPHAGES TO PRESENT OVA PEPTIDES TO OVA-SPECIFIC T CELL HYBRIDS

	Antigen (units/ml IL-2 production)			
Accessory cells	N-OVA	D-OVA	OVA tryptic peptides	OVA CNBr peptides
Live macrophages	160	160	320	320
Fixed macrophages	<20	<20	640	320
Live B lymphoma (A20)	1280	1280	640	1280
Fixed B lymphoma (A20)	<20	<20	320	320

gen recognition by T cells can occur within 1–2 hours following binding to the surface of the B cell. In contrast, rabbit anti-mouse Ig $F(ab')_2$ takes a considerably longer period of time and antigen recognition by RGG $F(ab')_2$-specific T cell hybrids does not occur until 5–6 hours following uptake (R. Chesnut and H. Grey, unpublished observations). Presumably this heterogeneity depends upon differences in the susceptibility of these proteins to degradation by lysosomal enzymes but further studies will be required to define the critical parameters of this heterogeneity.

In conclusion, when macrophages and B cells are compared by functional criteria, they behave in precisely the same manner to all the manipulations that have been used to support the concept of active metabolic processing events being a necessary aspect of antigen presentation to T cells. In rather striking contrast to this is the comparison between B cells and macrophages when the handling of antigen was examined biochemically. Whereas macrophages bind, endocytose, and degrade protein antigens very efficiently B cells perform the same functions 10- to 100-fold less efficiently. The one important exception to this statement is the efficient manner in which RAMIG was taken up and degraded by B cells. As mentioned above this exception may be of considerable importance in permitting antigen-specific B cells to efficiently process and present the antigen for which its mIg has specificity (and not present other antigens) to antigen-specific T cells. With respect to the non-Ig-mediated uptake of antigen by B cells, there is an apparent discrepancy between the capacity of B cells to process and present antigen on the one hand, and the relatively inefficient uptake, endocytosis, and degradation of protein antigens on the other hand. Two explanations for this apparent discrepancy might be invoked. First, there have been few efforts to quantitatively compare macrophages and B cells with respect to the

efficiency with which they present antigen. Studies by Cowing (49) suggest that macrophages are 4-fold more efficient on a per cell basis than activated B cells. This difference, however, may be an underestimate since normal macrophages are very heterogeneous with respect to Ia expression so that only a portion of the macrophages used in this study may have been functional APC. A second possible explanation for the apparent discrepancy between the functional capacity of B cells to process antigen and their limited ability to degrade antigen is that the degradation that is measurable (i.e., proteolysis to acid-soluble radioactivity) is to a great extent not relevant to the processing of antigen. It is known for instance, that the predominant end product of lysosomal degradation of proteins is free amino acids (50). This degree of proteolysis is clearly not relevant for antigen processing since the minimal size peptide that can be recognized by T cells is 7–9 residues (51, 52). Thus, the biochemical measurements of degradation may not accurately reflect the capacity of a given cell type to process antigen for immune recognition. Obviously more information about the detailed pathway of antigen processing must be acquired before these possible differences in antigen handling between macrophages and B cells can be evaluated.

IV. The Effect of B Cell Activation on APC Function

A. Comparison of Resting and Activated B Cells as Accessory Cells

The question of whether resting B cells can effectively present antigen to helper T cells or whether they must be activated before being capable of carrying out this function remains an important issue with regard to T–B collaboration and the delivery of helper signals to the B cell. Concern over this originally surfaced as a result of studies that investigated the ability of normal B cells and B cell tumors to present antigen to antigen-specific T cells (18). These studies centered on testing the ability of normal BALB/c B cells isolated from the spleens of nonimmunized mice and Ia^d-bearing B cell lymphomas to present KLH to an I-E^d-restricted KLH-specific T cell hybridoma. Somewhat surprisingly it was found that while the B cell lymphomas were quite effective at presenting KLH to the T cell hybridoma, normal B cells showed no antigen-presenting capacity except at the very highest cell numbers tested and appeared to be at least 50-fold less efficient than the B lymphoma cells. Because the B lymphoma cells were of a large lymphoblastoid phenotype, characteristic of activated B cells, the possibility that mitogen stimulated normal B lymphoblasts might be more like the B lymphoma cells and, thus, present antigen more effectively than nonactivated B cells was examined. The results confirmed this assumption and

demonstrated that indeed there were dramatic differences in the capacity of normal B cells and lipopolysaccharide (LPS)-B cells to present KLH. While as few as 100 LPS-B cells stimulated detectible IL-2 activity it required $>10^4$ normal B cells to present KLH with enough efficiency to detect T cell IL-2 production.

In contrast to these findings, Ashwell and co-workers published results showing that the antigen-presenting function of normal B cells was diminished by exposure of the B cells to γ-irradiation and that if high dose irradiation was avoided small resting B cells appeared to be as efficient as macrophages/dendritic cells at presenting antigens to T cells (53). With regard to the first observation Ashwell *et al.* reasoned that since other workers had used relatively large doses of γ-irradiation (3000–4000 rads) to block B cell proliferation prior to their being used as antigen-presenting cells, the previous inability to observe efficient antigen presentation by small resting B cells was probably a result of the affect of the irradiation on B cell function. As shown in Table IV, when different doses of irradiation, ranging from 500 to 3300 rads, were evaluated for their affect on the capacity of Percoll fractionated B cells to present GAT to a GAT-specific I-A^k-restricted long-term T cell clone the antigen-presenting capacity of the B cells began to decrease following exposure to 1500 rads with an even greater decline when the dose was increased to 2000 and 3000 rads. In contrast, when low-density Percoll

TABLE IV
EFFECT OF IRRADIATION ON DIFFERENT APC[a]

Sephadex G-10 B cells	Low-density cells	Irradiation to APC rads	T cell proliferation Δcpm
2×10^5	—	500	30,800
		1,000	29,600
		1,500	10,800
		2,000	2,200
		3,300	200
—	5×10^4	500	86,600
		1,000	88,000
		1,500	84,600
		2,000	68,600
		3,300	73,200

[a] A.5.1 T cells (2×10^4) were incubated with the indicated number of APC in the presence of GAT (100 μg/ml). T cell proliferation was calculated by subtracting this amount from the total amount of [^{3}H]thymidine incorporated (Δcpm).

fractionated cells, enriched for macrophages and dendritic cells, were exposed to the same dose of irradiation no decline in antigen-presenting function was observed. When the capacity of lightly irradiated (1000 rads) B cells obtained in this manner was compared with the heavily irradiated low-density cells containing macrophages and dendritic cells, no differences were observed in the ability of these two populations to present GAT (100 μg/ml) to the GAT-specific T cell clone, 11.4. Similar results were obtained when 1000 rad treated Percoll fractionated B cells were compared to 3300 rad treated normal spleen cells for their ability to present PPD to PPD-primed lymph node T cells. As stated above, the authors concluded from these results that small resting B cells were highly effective at presenting antigen to T cells and that, because of the unique affect of γ-irradiation on the antigen-presenting capacity of resting B cells, previous workers were misled in concluding that resting B cells were ineffective at presenting antigen.

In order to investigate the relative capacity of resting vs activated splenic B cells more extensively, our laboratory initiated a series of studies in which lightly irradiated (500 rads) splenic B cells were separated into four fractions on the basis of their buoyant density using Percoll gradient centrifugation (54). Cells isolated from 50/60, 60/65, and 65/72% Percoll interfaces and a >72% pellet were tested for their accessory cell function in three different assay systems, each making somewhat distinct demands on the accessory cells employed. The assay systems used included (1) the response of the I-A^d-restricted ovalbumin (OVA)-specific T cell hybridoma, 3DO-54.8, to intact native OVA; this presentation requires antigen uptake and processing prior to display of the appropriate epitope to the T cell. (2) The response of 3DO-54.8 to a tryptic digest of OVA which had been previously shown to contain the OVA peptide 323–339 which needs only to bind appropriately to the B accessory cell but does not require antigen processing prior to presentation (47, 48). (3) Con A stimulation of unprimed splenic T cells, a system in which there is neither genetic restriction nor a requirement for processing but which may require the elaboration of a costimulator activity such as IL-1.

Typical results are shown in Fig. 3 in which varying numbers of B cells from each of the fractions were tested for their accessory cell function. Although somewhat different quantitatively, the results with the three assay systems were qualitatively quite similar in that B cells from the lowest density fraction provided the greatest accessory cell function and that function declined progressively with increasing B cell density such that the B cells which passed through the 72% Percoll layer expressed little or no accessory cell function at any of the B cell concentrations tested.

While these data appeared to be in conflict with that of Ashwell and coworkers described above, it was found that by separating the cells as these

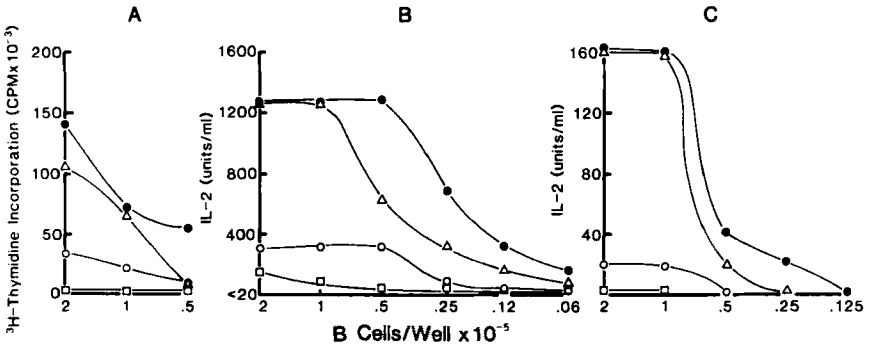

FIG. 3. Accessory cell function of Percoll fractionated splenic B cells. B cells obtained from the 50–60% (●), 60–65% (△), 65/72% (○), and >72% (□) fractions were used as accessory cells for a Con A-mediated splenic T cell proliferative response (A), as antigen-presenting cells for the presentation of OVA-tryptic digest (B), or antigen-presenting cells for the presentation of OVA (C). For B and C, the I-A^d-restricted, OVA-specific T cell hybridoma, 3DO-54.8 was used. B cells were exposed to 500 rads of γ-irradiation before use.

workers had done, namely isolating cells from a 60–70% Percoll interface, their findings could be reproduced. However, when the 60–70% population of cells was subfractionated by including a 65% Percoll layer, most of the activity was retained in the lower density 60–65% fraction suggesting that contamination of resting B cells with less dense activated B cells was the most likely explanation for the differences in the results.

Studies by Frohman and Cowing (49) using KLH-primed T cells and low dose irradiation of accessory cells resulted in similar conclusions. These workers estimated that LPS-activated B cells were approximately one-quarter as efficient as spleen adherent cells while a population of splenic B cells, enriched for resting B cells, were about 1/1000th as effective as spleen adherent cells.

The differences described above between resting and activated B cells and their ability to present antigen taken up nonspecifically may not be as apparent when the antigen is taken up via the membrane Ig (mIg) of the B cell. Recent studies in this area have been conducted by Tony and Parker (21) who have utilized RAMIG as the antigen and Ia-restricted NRGG-specific T cell lines or T cell hybridomas as the responding T cells. The population of cells enriched for small B cells was isolated from normal mouse spleens by counterflow elutriation techniques and tested for their ability to present as antigen F(ab')$_2$ fragments of rabbit anti-mouse IgM or IgD antibodies. They found that small B cells isolated in this fashion were as efficient as unseparated B cells in presenting the F(ab')$_2$ of RAMIG to the T cell hybrids. Their

results indicated that these responses were obtained with as little as 10–100 ng/ml of the rabbit antibody, an amount which by itself was insufficient to cause any discernible activation of B cells as measured by size change or Ia density increase. From this, the authors argued that resting B cells were capable of presenting this antigen to the T cell hybridomas and lines tested. However, because the T cells employed in these studies were proliferating T cells selected for stimulation by B cells and since during the course of the interaction between the B cells and T cells, these workers were able to show B cell proliferation as well as polyclonal Ig secretion, it was difficult to rule out the possibility that sufficient B cell activation took place following interaction with the RAMIG to allow an efficient presentation of antigen that perhaps would not otherwise have occurred. Recently these authors have provided some evidence that argues against this possibility in that they were able to demonstrate efficient presentation of antigen when the monovalent Fab′ fragments of RAMIG were used as the antigen (55). Since there has been no documentation of activation following administration of Fab′ anti-immunoglobulins, these data favor the authors' interpretation that in fact no activating signal need be administered via the mIg to allow for effective presentation of antigen when uptake of antigen is mediated by Ig receptors. However, as in the case of the $F(ab')_2$ RAMIG experiments the nature of the T cells used in these studies makes this interpretation tentative.

From the above data it appears that resting B cells are much less efficient than activated B cells at presenting antigen taken up by non-Ig-mediated means or at acting as accessory cells for a mitogen-stimulated response. How this difference relates to Ig-mediated antigen presentation by resting B cells remains less clear. The data described above using RAMIG $F(ab')_2$ suggest that resting B cells may be quite effective at presenting antigens taken up by their membrane Ig; however, a great deal more work needs to be done investigating the presentation of conventional antigens by resting antigen-specific B cells before any definitive conclusions can be drawn. In addition, T cells that are less highly selected than those used by Tony and Parker such as naive T cells or T cells that have not been recently primed will need to be studied.

B. Effect of Irradiation on the APC Function of B Cells

Since previous experiments had indicated that LPS-activated B cells were resistant to high doses of irradiation, the observations by Ashwell *et al.* indicating that the high-density B cells obtained from normal spleens were radiation sensitive suggested that B cells underwent a phenotypic change from radiation sensitivity to radiation resistance following activation. To explore this situation further, the radiation sensitivity of various macrophage-depleted B cells was studied (54). The results showed that at high dose

irradiation, all fractions failed to function as accessory cells, including the low density 50/60% fraction. This finding was in apparent contrast to that of Ashwell *et al.* who observed that the 50/60% fraction was radiation resistant. This can be explained, however, by the fact that in their studies macrophages/dendritic cells were not removed prior to Percoll centrifugation. When the irradiation-sensitive B cells from the >72, 65/72, 60/65, and 50/60% Percoll fractions were stimulated with LPS + dextran sulfate ($DexSO_4$), an increase in accessory cell function along with radioresistance was observed. By 48 hours all the fractions showed nearly equivalent levels of accessory cell function which by this time was completely resistant to 4000 rads of irradiation. Interestingly, when the kinetics of accessory cell function and radioresistance were tested, an increase in accessory cell function was observed which preceded the development of radiation resistance. Results of an experiment which illustrates the relationship of the antigen-presenting function to activation is shown in Table V in which the capacity of B cells from the 65/72% Percoll fraction to present OVA to the T cell hybrid 3DO54.8 was tested for up to 48 hours poststimulation with LPS and $DexSO_4$. Following exposure of the B cells to LPS and $DexSO_4$ for both 8 and 24 hours, although an increase in accessory cell function was observed, it was almost totally radiation sensitive whereas by 48 hours the antigen-presenting function was completely resistant to the affects of 4000 rads.

Taken together these data suggest that the capacity of B cells, freshly isolated from the lower density Percoll fractions, to present antigen in a radiosensitive manner is due to the fact that these cells are in some state of activation which allows them to express enhanced accessory cell functions

TABLE V
ACTIVATION OF B CELLS RESULTS IN ENHANCED ACCESSORY CELL FUNCTION WHICH IS INITIALLY RADIOSENSITIVE

LPS and $DexSO_4$ stimulation of 65/72% B cells (hours)[a]	3DO-54.8 T cell hybridoma response to OVA (IL-2 units/ml) γ-irradiation (rads)	
	500	4000
0	80	<20
8	160	<20
24	320	40
48	640	640

[a] Fractionated B cells were cultured in the presence of LPS and $DexSO_4$ for the times shown before γ-irradiation and used as accessory cells.

compared to small, high-density B cells, but that this function remains sensitive to high doses of irradiation. It is only at some later time after stimulation that the B cells become radioresistant in their APC function. It therefore seems clear that radiation sensitivity of B cell accessory cell function, per se, cannot be used as an indication that B cells are in a resting state.

Preliminary studies using cytofluorograph analysis of propidium iodide-stained cells to distinguish viable from dead cells suggest that differential rates of B cell death caused by exposure to 500 compared to 4000 rads may explain the radiosensitivity. However, the differences observed in the rate of cell death were not great and certainly other effects such as alteration in protein synthesis and peroxidation affects on lipids could influence the APC function of B cells. Recently Lowenthal and Harris (56) have published results using populations enriched for B cells which also suggest that resting B cells exposed to 10,000 rads are more rapidly killed than mitogen-activated B cell populations.

C. Mechanisms of Enhanced Accessory Cell Function by Activated B Cells

The reason why activated B cells are much more effective accessory cells than resting B cells remains controversial. Several possibilities exist for which data have been published, each of which, depending upon the nature of the antigen and the T cell population tested, may contribute, at least in part, to the enhanced capacity of activated B cells to present antigen.

One property which might affect the relative capacity of resting vs activated B cells to present antigen could be their ability to bind antigen. As described in Section III,A, it had been previously shown that normal B cells and B cell lymphomas differed in their capacity to bind and degrade intact protein antigens and that this difference appeared to correlate with their relative antigen-presenting capacity. However, it was not possible to determine from these studies if differences in antigen-presenting capacity were the result of differential antigen binding or differences between the various populations of B cells in their ability to process (i.e., internalize and degrade) and reexpress the processed antigenic moiety on the B cell surface.

To evaluate this question more directly the differences between normal B cells, activated B cells, and B lymphoma cells in their ability to bind and present an antigenic 17 amino acid OVA peptide was examined. A synthetic derivative of the 17 residue OVA peptide, containing a C-terminal Tyr, to enable radioidination, was added to freshly isolated splenic B cells, 3 day LPS + $DexSO_4$-stimulated B cells, or A20-1.11 B lymphoma cells, all of which had been previously fixed with paraformaldehyde to prevent endocytosis. After incubation for 1 hour at 4°C the cells were washed and the amount of ^{125}I-labeled peptide bound to the cells was determined. The

results of such an experiment are shown in Fig. 4. At any given peptide concentration, B lymphoblasts and A20-1.11 cells were able to bind approximately 5-fold more peptide than normal B cells. When a similarly pulsed population of B cells, activated B cells, and A20-1.11 cells was tested for its ability to present the OVA peptide to an OVA-specific T cell hybridoma, it was found that the activated B cells and A20-1.11 cells were efficient APC and stimulated 640 units/ml IL-2 which normal B cells stimulated only 40 units/ml IL-2. Thus, at least for this peptide, there was a direct correlation between the capacity of resting and activated B cells to bind the peptide and their capacity to functionally present the peptide to a T cell hybrid. These results would suggest the possibility that binding differences may be one of the mechanisms underlying the enhanced capacity of activated B cells to present antigen compared with resting B cells.

Another possible explanation for the differential capacity of resting B cells, activated B cells, and macrophages to present antigen has to do with the structure of the Ia molecule itself. While high-pressure liquid chromatography analysis has shown that the Ia molecules synthesized by spleen adherent cells and B cells have apparently identical polypeptide structures (57, 58), isolectric focusing studies have indicated the presence of differences which appear to be due to varying degrees of sialylation of the Ia α chain, the α chain from resting B cells having greater quantities than that from macrophages or activated B cells (57). As described above, Frohman and Cowing (49) have recently published results showing that Percoll fractionated splenic

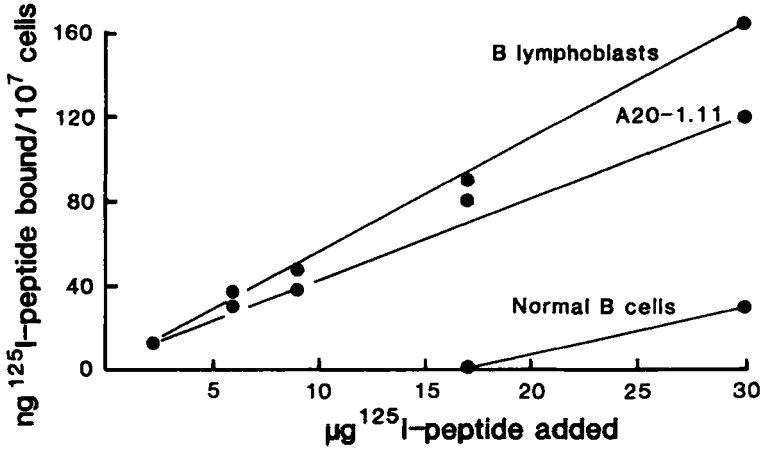

FIG. 4. Binding of the antigenic 17 amino acid OVA peptide to normal vs activated B cells. ^{125}I-labeled OVA peptide P323-339 was incubated with 10^7 lymphocytes for 1 hour at 4°C, washed, and the cell-associated radioactivity determined.

B cells enriched for resting B cells are approximately 250 times less efficient than LPS-activated B cells at presenting KLH to KLH-primed T cells. However, when the resting B cell fraction was treated with *C. perfringens* neuraminidase to remove sialic acid, a 24-fold increase in the antigen-presenting function of resting B cells was observed. This neuraminidase effect was observed only with the small B cell fraction; the capacity of spleen B cells enriched for large, low-density B cells, LPS-activated B cells, or spleen adherent cells to present KLH was not enhanced by neuraminidase treatment. More recent studies by Cowing and co-workers suggest that the Ia synthesized by mitogen-activated B cells is of the asialo form. This change in sialylation upon B cell activation was apparently specific for Ia and was not observed when other glycoproteins including class I MHC molecules were studied (Cowing and Hsu, personal communication). However, a final interpretation of these neuraminidase experiments will require isolation of Ia molecules from resting B cells, treatment of this purified Ia with neuraminidase, followed by functional studies comparing sialo with asialo Ia molecules incorporated into synthetic membranes to ensure that the critical effect of neuraminidase is on the Ia molecules themselves rather than on other membrane sialoglycoproteins.

Another change in Ia that occurs following B cell activation that might play a role in the enhanced APC function of activated B cells relates to the quantity of Ia expressed on resting and activated B cells. Several groups have reported that activation of B cells results in an appreciable increase in Ia expression. For instance, Mond and co-workers (59) demonstrated that *in vitro* culture of splenic B cells for 24 hours in the presence of anti-δ, anti-μ, or anti-κ antibodies, or their $F(ab')_2$ fragments, or *in vivo* administration of anti-δ antibodies iv into adult mice induced a 2- to 3-fold increase in the expression of surface Ia on B cells. Similarly, Monroe and Cambier (60) found a 4- to 5-fold increase in membrane Ia expression on B cells which had been stimulated with lipopolysaccharide and dextran sulfate. These workers observed that while the increase in Ia density was expressed during the transition of cells from the G_0 to the G_1 phase of the cell cycle, there was a decrease in membrane Ia expression from this peak level as the cells progressed through S, G_2, and M phases. Since the increased level of Ia expression appeared to coincide with the time when the B cells were first becoming activated, these authors suggested that the increased expression of Ia might serve an important function in directing Ia-restricted, antigen-specific T cell help for thymus-dependent antigen-driven B cell activation.

How the level of Ia expressed on accessory cells might influence APC function has been examined by several groups of investigators. Matis and co-workers demonstrated that under conditions of limited antigen concentration, the number of Ia molecules expressed on the antigen-presenting cell

directly influenced the magnitude of the T cell response (61). The presentation of low concentrations of pigeon cytochrome *c* (2.4 n*M*) to an anti-I-E^k-restricted cytochrome *c*-specific T cell clone by splenic antigen-presenting cells expressing one, one-half, or one-eighth the level of I-E^k resulted in proliferative responses that were inversely proportional to the level of Ia expression (i.e., 75,000, 40,000, and 5,000 cpm, respectively). It was further found that the low expression of Ia could be compensated for by a proportional increase in the antigen concentration. Thus, using antigen-presenting cells with one-half the level of Ia an optimal T cell response could be generated by using twice the amount of antigen used with antigen-presenting cells that expressed a 1× level of Ia. At high antigen concentrations, however, the influence of Ia expression on the level of T cell activation was not apparent.

Using a somewhat different approach, Tite and Janeway arrived at a similar conclusion (62). Employing cytolytic ovalbumin-specific Ia-restricted Lyt-1^+2^-, L3T4a^+ cloned T cells which in the presence of ovalbumin were cytotoxic for B cells, and a series of sublines of the B cell lymphoma A20.2J, selected for expression of different levels of Ia molecules, these workers found that at suboptimal antigen concentrations the level of B cell killing was directly dependent upon the amount of Ia expressed on the B cell; furthermore, they found the T cells to be cytotoxic for activated B cells but not for resting B cells.

Recent studies by Roehm and collaborators (63) have also addressed this question. Using a combination of soluble factors derived from the macrophage tumor line $P388D_1$ and a Con A-stimulated T cell hybridoma, they were able to induce as much as a 14-fold increase in the level of Ia expression. Their results showed that those B cells which had been induced to express high levels of Ia were considerably more effective at presenting antigen than noninduced B cells.

The problem with the three approaches described above is that while it is evident that there are differences in the level of expression of Ia on the various antigen-presenting B cell populations, it is impossible to determine if there are concomitant differences, either qualitative or quantitative, in the expression of non-Ia molecules which may also be important for T cell interaction and for which we do not yet have a means of detection. In an effort to circumvent these problems our laboratory has utilized the recently developed technique of constructing an artificial antigen-presenting surface composed only of purified Ia molecules incorporated into a cholesterol and phospholipid matrix. Liposomes prepared with these lipids and Ia can be coated onto glass beads and used to present the previously described ovalbumin peptides to Ia-restricted ovalbumin-specific T cell hybridomas. By varying the amount of Ia incorporated into the synthetic membrane and the amount of antigenic peptide added to the culture the influence of Ia density and

antigen concentration can be evaluated independent of other macromolecules. Shown in Fig. 5 is an example of the results that were obtained in carrying out such a study. On the ordinate is plotted the nanograms of IA^d detected on the liposome-coated glass beads as determined by the binding of ^{125}I-labeled monoclonal anti-IA^d antibody and on the abscissa is plotted the nanograms of the OVA peptide antigen added to the culture. The data shown depict the concentration of Ia and antigen required to induce a detectable quantity (20 units) of IL-2 by the responding T cell hybridoma. The results indicate that at low antigen concentrations the ability to effectively present antigen to T cells is inversely proportional to the Ia density. Thus, with 100 ng of antigen, approximately 6-fold less Ia is required than with 20 ng of antigen. This finding is a direct confirmation of the results of the studies described above using intact cells as the antigen-presenting surface and demonstrates that when the concentration of antigen is limited, the density of Ia molecules alone can significantly affect the ability of a cell to present antigen.

Our laboratory has recently extended this type of experiment to explore the defect in the antigen-presenting cell function of resting B cells. Shown in Fig. 6 are the results obtained when the $I\text{-}A^d$ molecules isolated from normal splenic B cells were compared with the $I\text{-}A^d$ isolated from the B cell lympho-

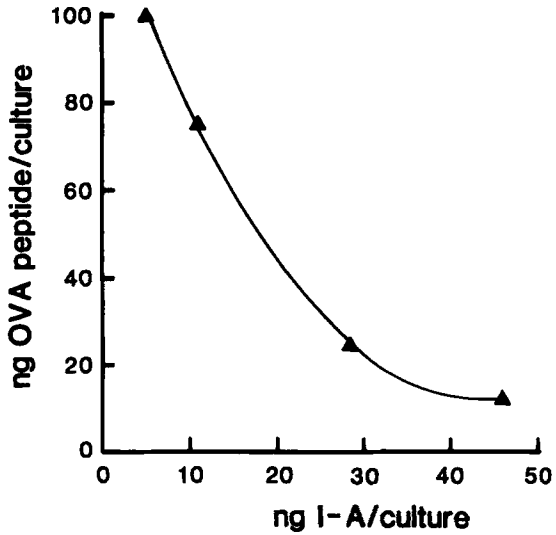

FIG. 5. The relationship between Ia density and antigen concentration to antigen presentation by glass beads coated with Ia-containing liposomes. Each data point represents the minimal peptide concentration required to give a detectable stimulation of the T cell hybridoma (20 U IL-2/ml) with a particular concentration of Ia.

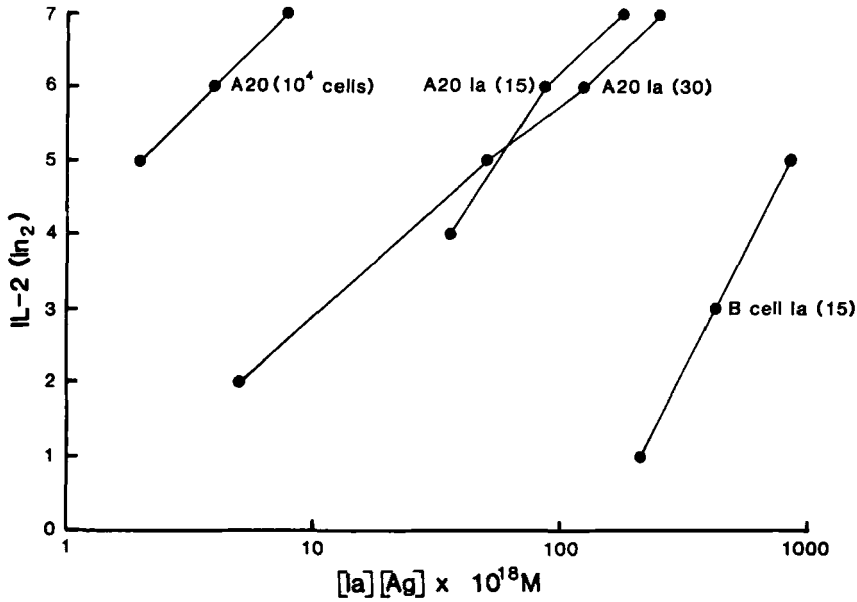

FIG. 6. A comparison of the antigen-presenting capacity of liposomes containing I-A isolated from normal B cells or A20 B lymphoma cells vs intact fixed A20 B lymphoma cells. The numbers in parentheses represent two different bead preparations constructed with liposomes formed with two different concentrations of Ia (15 and 30 μg/ml).

ma, A20-1.11, and the I-A^d present on intact fixed A20-1.11 cells. The results are plotted as the product of I-A expressed on the liposome-coated glass beads or A20-1.11 cells (i.e., molar concentration of IA) and by the molar concentration of antigenic peptides vs the quantity of IL-2 ($\log_2$) produced by the responding T cell hybridoma. By plotting the product of the Ia and antigen concentrations the inverse relationship between antigen and Ia concentration described above was normalized thus allowing a direct comparison of the efficiency of the I-A^d molecules from different sources in mediating antigen presentation. The data illustrate two points. First, there is approximately a 10- to 20-fold difference in the ability of Ia isolated from normal splenic B cells, and the Ia isolated from A20-1.11 cells, in eliciting an IL-2 response from the OVA-specific T cell hybrid. Although there are certainly other possible explanations for these results they suggest that there is a structural difference between the Ia from normal B cells and the B lymphoma cells which allows the latter to be more effective at presenting antigen. Such structural differences could be related to the carbohydrate structure on the Ia molecules as suggested by the studies of Cowing and co-

workers (49) described above, or other differences in the protein structure which have thus far gone undetected using monoclonal antibodies and two-dimensional gel electrophoresis analysis.

The second piece of information illustrated by this figure has to do with the relative effectiveness of Ia bearing liposome-coated glass beads and intact fixed cells at presenting antigen. When fixed A20-1.11 cells were compared to the I-A which had been isolated from A20-1.11 cells and incorporated into liposomes, the I-A expressed on intact A20 cells was approximately 20- to 30-fold more effective at eliciting an IL-2 response than the purified A20-1.11 I-A expressed on liposome-coated glass beads. These results suggest that there are characteristics about the antigen-presenting cell surface other than Ia structure which dramatically enhance the ability of A20-1.11 cells to act as an antigen-presenting cell. These differences could be related to differences in the mobility of Ia molecules in the plane of the membrane of intact cells which may be very different than that of Ia molecules that are expressed in the synthetic lipid bilayer; alternatively other molecules expressed on accessory cells but not present in the liposomes which have yet to be identified may also be involved in enhancing the effectiveness of intact cells. Such molecules might act by enhancing the adhesion between the antigen-presenting cell and the responding T cells or in other ways promote the triggering of the T cell to produce IL-2. While not being absolutely required for antigen presentation, non-Ia molecules could also function to stabilize or maximize antigen and Ia interactions. If these auxiliary molecules exist it is possible that activation of B cells could enhance their expression which could also increase the antigen-presenting function of activated B cells compared to resting B cells.

Another possible factor responsible for the difference in accessory cell function between resting and activated B cells could be the differential capacity to provide a costimulator, IL-1-like signal to the responding T cells. While the exact role(s) of IL-1 in T cell activation remains controversial, it does appear to function in the elaboration of IL-2 and, in addition, may influence the expression of IL-2 receptors on the T cell surface (64). Although efforts by our laboratory as well as those of other investigators (49) to detect the presence of IL-1 activity in the culture supernatant of either resting or activated murine B cells or B cell lymphomas has failed to detect such an activity, Oppenheim and co-workers have recently demonstrated the presence of an IL-1-like activity produced constitutively by Epstein–Barr virus-transformed human B lymphocytes and by normal human peripheral blood B lymphocytes following stimulation with lipopolysaccharide (65, 66; and Matsushima and Oppenheim, personal communication). The physical properties of the IL-1-like activity elaborated by the human B cells were similar to those for monocyte derived IL-1 and rabbit anti-monocyte-

IL-1 antibodies were able to block the activity of B cell-derived IL-1 like activity in the thymocyte proliferation assay.

Recent experiments by Kurt-Jones *et al.* (67) have suggested that murine B cells may express a membrane bound form of IL-1-like activity similar to that previously described by these workers in macrophages. Using an IL-1-dependent T cell line (D10.G4.1) these workers found that B cells which had been activated with mitogens plus T lymphokines could support the Con A-stimulated growth of D10.G4.1 cells presumably by providing a source of membrane bound IL-1 like activity.

V. The Capacity of B Cells to Function as Stimulator Cells in Mixed Lymphocyte Proliferative Responses

The presentation of allo-Ia antigen has been studied extensively using the mixed lymphocyte response (MLR) as an indicator of alloantigen recognition. Despite the well-characterized expression of Ia by B cells there has been considerable controversy over the ability of these cells to act as MLR stimulator cells. Glimcher and co-workers showed that several B cell tumors could stimulate a MLR although not all Ia^+ tumors apparently could carry out this function (15). The earliest studies using normal lymphoid cells that attempted to define the active stimulator cell suggested that B cells were capable of MLR stimulation (68–71) although subsequent results using improved techniques for removal of macrophages and dendritic cells suggested that B cells were not stimulatory (72–77). While recent investigations clearly demonstrated the ability of both macrophage and dendritic cells to stimulate a primary allogeneic T cell response, the apparent failure of Ia^+ B cells to stimulate a MLR suggested that the expression of Ia antigen on the cell surface, although necessary, is not sufficient for allogeneic T cell stimulation.

Since previous studies had shown that activated B cells were much more effective than resting B cells at presenting conventional antigens to antigen-specific MHC-restricted T cells our laboratory has reinvestigated the ability of B cells to stimulate MLR responses (78). For the initial experiments two sources of previously primed alloreactive T cells were used, namely T cell hybridomas and a heterologous population of short-term *in vitro* propagated alloreactive T cell lymphoblasts. Our findings demonstrated the following: B cells cultured for 3 days in LPS plus $DexSO_4$ were equivalent to normal spleen cells in their ability to present allo-Ia to the T cell hybridomas as measured by IL-2 production. When normal splenic B cells were analyzed in this regard, B cells obtained from low-density Percoll fractions were more effective at stimulating an alloreactive T cell response than B cells isolated from the high-density Percoll fractions. Similar results were obtained using the *in vitro*-propagated T lymphoblasts. These findings demonstrated that B

cells could at least stimulate a secondary MLR and that although there may be differences in the efficiency of MLR stimulation between resting and activated B cells both populations appeared to be capable of properly presenting the allo-Ia antigen to alloreactive T cells.

These findings have recently been confirmed by two other groups of investigators. Inaba and Steinman (79) estimated that B lymphoblasts were 30–50% as efficient as dendritic cells while normal spleen B cells were 5–20% as effective as dendritic cells at stimulating *in vitro*-propagated alloreactive T lymphoblasts. Minami and co-workers (80) found that both normal splenic B cells and LPS-stimulated B lymphoblasts could stimulate some but not all alloreactive T cell hybridomas. These workers also found that the LPS-B cells were superior to normal B cells in stimulating IL-2 production by the T cell hybridomas. The results of this study are described in greater detail in Section VI.

Although able to stimulate secondary responses, freshly isolated normal B cells appear to lack the ability to stimulate a primary MLR. Furthermore, when stimulated by B cell growth factor (BCGF) to produce large quantities of Ia, B cells were also unable to stimulate a MLR. In contrast, B cells stimulated for 3 days with LPS and dextran sulfate (L/D) were potent stimulators of a primary MLR. Since Ia expression on BCGF- and L/D-stimulated cells were similar, it was concluded that the capacity of 3-day L/D-activated cells to stimulate a MLR was due to the elaboration of necessary costimulator molecules that the BCGF-stimulated cells lacked. IL-1 did not appear to be the costimulator involved, since L/D-activated cells failed to express IL-1 activity as measured by the sensitive D10.G4.1 assay system. This is consistent with the finding of Kurt-Jones *et al.*, who found IL-1 expression by B cells required a mitogen plus T cell-derived factor(s). Furthermore, recombinant IL-1 added to MLR cultures containing BCGF-stimulated B cells failed to function as a costimulator. In contrast, the phorbol ester, PMA was a potent costimulator in this system. We concluded from these experiments that appropriately activated B cells (3-day L/D cells) can function as stimulators of a primary MLR and that they elaborate critical costimulator molecules which appear to be distinct from IL-1, that enable them to function in this regard.

Similar to the finding with antigen presentation, the ability of normal splenic B cells to stimulate a MLR was sensitive to the affects of γ-irradiation. For example, freshly isolated B cells irradiated with 500 rads were able to stimulate allo-Ia-specific T cell hybridomas to secrete IL-2 (320–640 units/ml) while the same preparation of B cells following exposure to 4000 rads was unable to stimulate a detectable level of IL-2. However, the capacity of 3 day LPS + $DexSO_4$-stimulated B cells to stimulate alloreactive T cell

hybridomas (640 units/ml IL-2) was not diminished by exposure to 4000 rads.

It is also conceivable that the relative inefficiency of resting B cells to stimulate a MLR involves the allorecognition not only of Ia but of some other non-Ia component as well, which is poorly expressed on the surface of resting B cells (allo Ia + X). To address this possibility, our laboratory has tested the capacity of purified Ia molecules to stimulate alloreactive T cell hybrids using the liposome-coated glass bead system described in Section IV,C. Of the 10 hybridomas tested, 8 (4 I-A^d restricted and 4 I-E^d restricted) responded to the appropriate purified Ia molecule in the absence of other cell membrane proteins. These findings suggest that the majority of alloreactive T cells recognize class II MHC molecules alone and do not recognize allo-Ia + X. Thus, the difference between resting and activated B cells in their capacity to stimulate a MLR is not likely to be due to the failure of resting B cells to adequately express an antigen other than Ia that is corecognized with Ia by alloreactive T cells.

Although the most frequently studied allogeneic mixed lymphocyte responses have been those directed against MHC-encoded determinants, at least in mice, a large primary T cell proliferative response can also be mediated by gene products of the non-MHC-linked minor lymphocyte stimulating (Mls) locus first described by Festenstein and which has been mapped to chromosome 1 (82). Four alleles have been described for the Mls locus; Mls^a and Mls^d are cross-reactive and stimulate a strong T cell proliferative response while Mls^b and Mls^c stimulate much weaker responses. To date, no antigen coded by the Mls locus has been characterized and numerous attempts to produce monoclonal antibodies which would identify the antigen involved have proven unsuccessful. It has been shown that B cells but not T cells express the determinants responsible for the Mls MLR (83, 84). In a series of investigations Ahmed, Scher, and co-workers (85, 86) studied the expression of Mls activity in the ontogeny of murine B cell development. Using fluorescence-activated cell sorter (FACS) analysis and sorted populations of cells stained with anti-Ig and complement receptor antibodies these workers showed that the expression of Mls activity defined a subset of relatively mature B cells which first appeared in the spleens of mice at about 2 weeks of age and reached adult levels by about 4–5 weeks. By examining the surface phenotype of the various B cell subsets it was deduced that Mls activity appeared after the expression of IgM, IgD, Ia, and complement receptors at about the time of expression of the differentiation marker Lyb-5.

Webb and co-workers (87) using Percoll density gradients have subdivided the B cells obtained from the lymph nodes of normal mice into two populations, one enriched for small dense B cells and a second enriched for

large, low-density B cells. When these B cell fractions were tested for Mls MLR stimulating capacity it was found that the cells from both fractions stimulated strong Mls responses, equal to or better than an unseparated spleen cell population with the low-density large B cells being approximately 4-fold more efficient than the small dense B cells. The observation that the fraction enriched for small B cells was effective at stimulating a primary Mls MLR is in apparent contrast to our own findings in which small B cells obtained from the >72% Percoll fractions were >10-fold less efficient than activated B cells in stimulating a primary Mls MLR, although these same B cells were quite efficient in stimulating Mls-specific T cell hybrids. The basis of this difference is not known but is probably a reflection of different degrees of contamination with activated B cells in the different preparations.

The role of Ia antigens in the Mls MLR has also been controversial. Janeway and co-workers published evidence suggesting that the primary response to the Mls locus was restricted to self-Ia antigens and that this response could be blocked by conventional, as well as monoclonal, anti-I-A antibodies (88). However, these workers found that the secondary responses did not show MHC restriction. In contrast to these findings Molnar-Kimber and Sprent found no evidence for H-2 restriction for a primary or secondary Mls response (89). In addition, Webb *et al.* (90) using Mls-reactive T cell clones found no evidence for MHC restriction using a variety of MHC haplotypes as stimulator cells. Using an $Mls^{a,d}$-reactive T cell hybridoma (D1G10.G11) we also found the response to be totally non-MHC restricted (Chesnut and Grey, unpublished observation).

Although it seems clear that B cells can stimulate an Mls MLR the ability of other cell types to act as Mls stimulator cells is more difficult to assess. Several groups (91–93) have published results showing that either peritoneal macrophages or spleen adherent cells, in the latter case specifically depleted of B cells, could stimulate at least some degree of Mls-mediated T cell proliferation. In the case of spleen adherent cells the capacity of this population to stimulate an MHC-mediated MLR was dramatically better than their ability to stimulate an Mls MLR (93). Preliminary studies have shown that, in contrast to their potent MHC MLR stimulating capacity, mouse dendritic cells are unable to stimulate a Mls MLR. However, more studies with defined cell populations are needed before any conclusions on the relative capacity of B cells and other cell types to stimulate a Mls MLR can be made.

In light of the above data, it remains uncertain as to whether the Mls antigen is expressed uniquely on B cells or whether like Ia molecules it may be restricted in its expression but present on a number of different cells depending upon their state of differentiation or activation. In addition, we still do not know what role, if any, the Mls antigen plays in B cell function

and how this antigen is able to stimulate such a strong T cell proliferative response.

From the data discussed in this section, we know that normal as well as activated B cells can stimulate both an MHC class II- and an Mls-mediated MLR; although resting B cells appear to be much less efficient as stimulators of both an MHC MLR as well as an Mls MLR. Certainly in the future it will be important to characterize the Mls antigen(s) in order to understand its role in T–B collaboration as well as any possible relationship to MHC-mediated T–B interactions. Hopefully it will be possible to dissect the Mls MLR as is currently being done with the MHC MLR by examining the stimulation of alloreactive T cells using isolated Ia molecules incorporated into synthetic membranes, which should allow us a clearer view of B cell Mls stimulation.

VI. Differences in the Accessory Cell Function of B Cells Compared to Other Cell Types

Although the studies described above clearly demonstrate the capacity of B cells to take up, process, and present antigen in a manner analogous to that of other accessory cells such as macrophages, there is growing evidence that there may also be differences between B cells and macrophages/dendritic cells when particular T cell antigen presentation systems are examined in detail. The information regarding this point is quite recent and it is likely that many more examples of differences between B cells and non B accessory cells will be documented in the future besides those that are discussed below. Three distinct but somewhat related areas regarding this matter have been investigated: first, differences in the signals evoked from T cells by B cells and macrophages even though both types of cells can present the antigen to T cells; second, differences in the capacity of B cells and macrophages to appropriately process and/or present antigen as reflected by differences in their capacity to stimulate T cells; and third, differences between distinct B cell clones to process antigen and generate a particular T cell-recognizing epitope that is dependent on the individual antigenic determinants recognized by the B cell mIg.

A. Differences in the Extent of Stimulation of T Cells When B Cells and Non-B Cells Are Used to Present Antigen

Recent studies from three laboratories have suggested the possibility that B cells, although capable of presenting antigen to T cells, may not deliver all of the activating and differentiation signals to T cells that can be provided by

other accessory cells. One of the more definitive studies that provides evidence for this type of distinction between B and non-B accessory cells was performed by Ashwell *et al.* (53) in their experiments on the capacity of resting B cells to present antigen to T cell clones and T cell hybrids. Two different assays were used to measure T cell stimulation: (1) the capacity of B cells to stimulate T cells to proliferate (and/or elaborate IL2), and (2) the capacity of B cells to stimulate T cells to elaborate a factor(s) which caused the proliferation of the antigen-presenting B cells or bystander B cells (i.e., a B cell growth factor). With the majority of the T cell clones analyzed, B cells and macrophages behaved similarly and induced the elaboration of the lymphokines required for both T cell proliferation and B cell proliferation. However, in at least one instance, a T cell clone specific for the antigen GAT, was stimulated by B cells to produce the factor(s) required for B cell proliferation but was not itself stimulated to proliferate or produce IL-2. In contrast, macrophages were capable of inducing this same T cell clone to produce IL-2 and proliferate in addition to making the lymphokines required for B cell proliferation. These data suggest that B cells and macrophages/dendritic cells may differ in the manner in which they signal T cells and that B cells may in some cases be incomplete T cell stimulators, capable of inducing T cells to elaborate some lymphokines but not others.

Erb and co-workers (94, 95) studying the capacity of B cell tumors to present KLH to KLH-primed T cells concluded that the B cell tumors lacked the ability to induce T helper function in a population of T cells primed 1 month previously with antigen, but could induce helper function if the T cells were recently primed *in vivo* or were *in vitro* propagated. Thus, it was postulated that the B cell tumors' capacity of effectively trigger T cell help was limited to circumstances in which the T cells had been recently stimulated and thus did not require the elaboration of some stimulatory factor that was necessary for the activation of "memory" T helper cells. Although these experiments are of interest and intriguing, there are certainly other possible interpretations to explain the apparent differences between the functional capacity of B cells and macrophages in these experiments.

The third study to suggest that B cells may be somewhat limited in their signal capabilities to T cells was performed by Sercarz and collaborators (96). Using C57BL/10 mice that are low responders to the antigen hen egg lysozyme (HEL), antigen-specific T cell lines and clines were derived. When these T cells were used as helper cells for the plaque-forming cell response of HEL-primed B cells, no enhanced response was observed unless normal T cells or soluble factors derived from Con A-stimulated spleen cells were added to the cultures containing antigen-specific T and B cells. In contrast, when the same experiments were performed with high responder H-2^d

mice, then enhanced antibody formation was obtained in the absence of any additional soluble factors. These results were interpreted to suggest that the B cells from low responder H-2^b mice, although they displayed antigen appropriately, did not stimulate T cells to elaborate the lymphokines needed to cause B cells to differentiate to antibody-secreting cells. These lymphokines had to be provided by normal T cells or Con A suppressor cells. In contrast, the high responder H-2^d B cells could provide all the signals required for the stimulation of T helper cells.

B. Differences between B Cells and Other Accessory Cells in Their Capacity to Process and/or Present Antigen

In the preceding section, the inability of B cells to stimulate T cells in an antigen-specific manner was evident in certain T–B interaction systems but not others. The interpretation placed on these results was that although B cells could display antigen appropriately they did not deliver all the required signals for the stimulation of T cells to elaborate certain lymphokines detected by some of the assays. The alternative possibility, that the B cells did not process and/or present the antigen appropriately, was evaluated and ruled out in some systems but not in others. In this section experimental data to suggest that there are circumstances that distinguish B cells and macrophages/dendritic cells in their capacity to process and present antigen will be assessed. Unfortunately, in some of these experiments alternative interpretations can be offered to explain the data. The most convincing studies that provide evidence for defects in antigen processing and/or presentation by B cells come from experiments utilizing T cell hybrids since these cells are stimulated to produce IL-2 by recognition of antigen/MHC alone and do not require other accessory cell-dependent interactions and/or factors to stimulate them. The strongest data in support of this conclusion are the capacity of purified Ia (when present on large surfaces afforded by glass coverslips or glass beads) to present antigen when suitable antigen-derived peptides are used as the antigenic stimulus for triggering the T cell hybrids (97, 98).

A striking difference between B cells and non-B cells in their capacity to serve as APC for alloreactive T cell hybrids was observed in the study by Minami *et al.* (80) alluded to in Section V. In this study, 134 T cell hybrids derived from a fusion of a primary mixed lymphocyte culture between H-2^b stimulator cells and H-2^k responder cells were analyzed. One hundred twenty-five of the hybrids were specific for the I-A^b antigen. When non-B splenic accessory cells were used (presumably containing both macrophages and dendritic cells), all of the hybrids were shown to respond by the elaboration of IL-2. When purified normal B cells or LPS-activated B cells were used as

stimulator cells, only 69 of the 125 hybrids responded. The differences did not appear to be quantitative in nature, in that there were highly reactive T cell hybrids that responded only to macrophage/dendritic cells and relatively poorly reactive hybrids that responded to both B cells and macrophage/dendritic cells, and vice versa. Thus, approximately half of the T cell hybrids analyzed responded to macrophage/dendritic cells as well as B cells whereas half were specific for macrophages/dendritic cells and were unresponsive to the I-A antigens presented by B cells. The nature of this difference is unknown and whether it represents a structural difference between the I-A molecules expressed on B cells and non-B cells or whether it reflects other requirements for T cell recognition in this system remains to be elucidated. Our laboratory has made a somewhat similar observation with a collection of alloreactive T cell hybrids that we have analyzed. In these experiments, the hybrids were initially screened on Ia^d B cell tumors as stimulator cells. As a consequence all of the hybrids subsequently studied were reactive with B cells although several of these hybrids did not react with Ia^d splenic macrophages/dendritic cells. Furthermore, we have observed some heterogeneity between different B cell tumors in their capacity to stimulate these alloreactive T cell hybrids. When two different B lymphoma lines were compared for the capacity to stimulate alloreactive hybrids, examples were found in which one of the lymphomas was an efficient stimulator whereas the other was not. Again, whether this difference reflects structural heterogeneity of the Ia molecules derived from different B cell tumors or whether there are other molecules involved in the stimulation of these T cell hybrids that are distinct from Ia is at present unresolved.

Studies with conventional protein antigens also suggest differences between B cells and non-B cells in their capacity of process and/or present antigen. Ashwell *et al.* (53) compared the capacity of B cells with that of macrophage/dendritic cells in presenting the antigens cytochrome *c*, hen egg lysozyme, and GAT to 13 T cell clones and 4 T cell hybrids. Utilizing both the T cell clones and the hybrids, it was found that although most of them responded to both macrophage/dendritic cells as well as B cells, there were some instances in which only macrophage/dendritic cells were effective APC. Thus, with 4 of 13 T cell clones and 1 of 4 T cell hybrids, a response was obtained only with non-B accessory cells. Obviously the interpretation of these data is more complex than that dealing with alloreactive cells where the only recognition required presumably is of the Ia antigen itself. Thus, it is not possible at the present time to determine whether the failure of B cells to effectively present antigen to some of these T cell clones was due to the failure to appropriately process the antigen by the B cells or whether the MHC-restriction element produced by the B cells was different than that produced by macrophages/dendritic cells and was not recognized

by those T cells that failed to be stimulated by B cells. Also, as mentioned above, the negative data with the four T cell clones (but not the T cell hybrid) could be due to a failure of the B cells to provide some necessary costimulator activity rather than representing a defect in antigen recognition.

Further evidence that B cells may not be capable of processing all antigens in an efficient manner has come from an analysis of T–B interactions utilizing T cell hybrids specific for the antigen beef insulin (99). The T cell assay system used in these studies was the provision of help to normal B cells for a polyclonal antibody response as measured by a reversed plaque-forming cell assay. T cell hybrids specific for either the A chain or the B chain of insulin were studied. When the intact beef insulin molecule was used as the antigen in this system, A chain-specific T cell hybrids provided efficient help for the polyclonal PFC response, whereas, the B chain-specific T cell hybrids were completely ineffective in providing T cell help. However, this defect in B chain stimulation could be corrected by using isolated B chains rather than intact beef insulin as the antigen. Testing of B cells from BM12 mutants demonstrated the requirement for histocompatibility in the I-A subregion, which was not correctible by the addition of H-2^b non-B accessory cells, thus demonstrating the requirement for histocompatibility between T and B cells in this response. This is consistent with the concept that B cells were serving as antigen-presenting cells and that there was a defect in the processing of intact insulin but not isolated B chains by the B cells. Utilizing these same antigens and assaying IL-2 secretion by the T cell hybrids, it was demonstrated that the same B chain-specific T cell hybrids could respond to B chain in the context of the intact beef insulin molecule when macrophages were used as antigen-presenting cells further supporting the notion of a defect in antigen processing by B cells in contrast to macrophages. A more recent study by the same workers (100) has utilized these hybrids in an antigen-specific response using DNP insulin and isolated A and B chains with the same T cell hybrids. As was shown in the polyclonal response, a specific anti-DNP response was obtained only when the B chain-specific T cell hybrids were used in conjunction with DNP-isolated B chains and not intact DNP beef insulin.

C. The Relationship between the Epitopes Recognized by B Cells and Their Capacity to Interact with Antigen-Specific T Cells

The question that is addressed in this section relates to whether B cells that take up antigen via their immunoglobulin receptors allow for the expression of all T cell-specific antigenic determinants similarly or whether the binding of the antigen to different B cells possessing surface immu-

noglobulins of distinct epitopic specificities influences the manner in which antigen processing occurs. If the latter were the case it would be expected to result in the preferential presentation of certain T cell antigenic determinants over others. This hypothesis, initially proposed by Berzofsky (101) and termed T–B reciprocity, was based on several pieces of indirect data that suggested that there was a specific relationship between the epitopic specificity of the B cell and that of the T cell that was necessary to obtain effective T–B collaboration. For instance, preliminary data by Berzofsky suggested that T cells specific for a particular peptide fragment of myoglobin provided help preferentially to B cells that recognized antigenic determinants present on the same peptide fragment. Recently, Manca *et al.* (102) have provided somewhat more direct evidence suggestive of the same model. In this study, the antibody response to β-galactosidase was analyzed utilizing normal B cells and T helper cells specific for distinct cyanogen bromide peptides derived from the β-galactosidase protein.

Three measurements of antibody production were made. The total antibody response was measured in an ELISA system that determined the binding of antibody to the β-galactosidase protein. The second assay measured antibodies specific for unique determinants on β-galactosidase that, when bound by antibody, made the enzyme resistant to heat denaturation. The third type of antibody enabled mutant β-galactosidase molecules that were enzymatically inactive to become active following combination with these antibodies. Critical to the interpretation of these results was the previous demonstration, using monoclonal antibodies, that the antibodies and the determinants for which they were specific that protected β-galactosidase against heat denaturation were distinct from the antibodies and the determinants involved in the reconstitution of the enzymatic activity of mutant galactosidases. When T cells specific for different cyanogen bromide peptides within galactosidase were compared with one another with respect to their efficiency in providing help as measured by these three different antibody assays, strikingly different results were obtained as illustrated in Fig. 7. The T cells most efficient for eliciting a total antibody response were those immunized with either intact denatured galactosidase (RCMGZ) or fragment CB18, whereas CB20 which was relatively inefficient for eliciting help as measured by total antibody production was the most efficient immunogen for eliciting help in the production of antibodies involved in the protection of β-galactosidase from heat denaturation. In contrast, the CB18-specific T cells were very inefficient in this regard. A third source of T helper cells immunized to CB21 were the most efficient source of helper cells for the induction of antibodies involved in the reconstitution of the enzymatic activity of the mutant enzyme (Fig. 7b). Also, studies were performed utilizing different mutant enzymes and testing the capacity of the different sources of

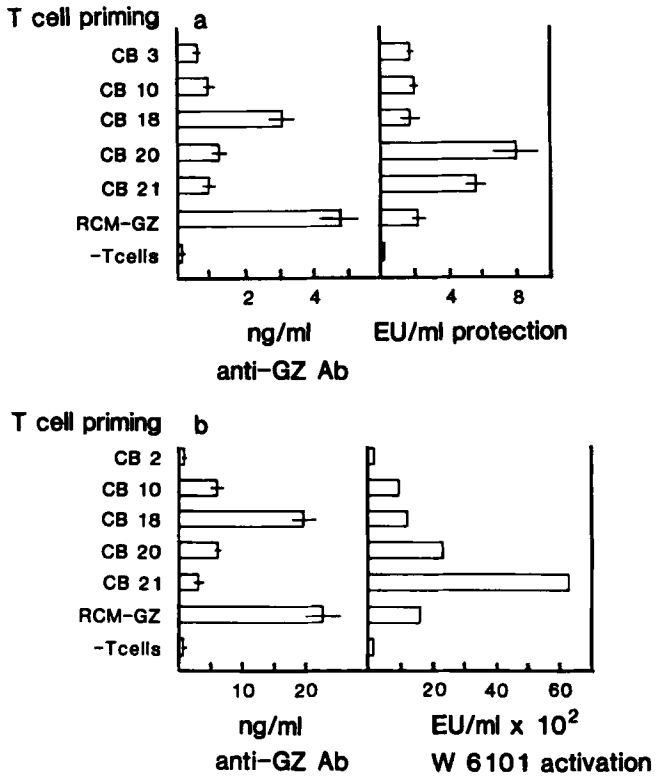

FIG. 7. Peptide-primed T cells provide help for production of both activating and protecting antibodies. (a) The production of protecting antibodies. Lymph node T cells from mice primed with reduced and carboxy-methylated β-D-galactosidase (RCM-GZ) or various cyanogen bromide (CB) peptides were cultured with B cells in the presence of native GZ. (b) The production of activating antibodies under similar culture conditions.

T cells to provide help for the induction of reconstituting antibodies. In four of the five mutants studied the CB21 immune T cells provided the best source of helper activity. In contrast, T cells immunized with CB20, a peptide which was quite inefficient in inducing antibodies with the capacity to reconstitute the enzymatic activity of the other four mutants, was the most efficient helper cell source for the induction of reconstituting antibodies for the fifth mutant.

Sercarz's laboratory has made similar observations in the galactosidase system but, rather than measuring the immune response to distinct galactosidase antigenic determinants, they measured the capacity of different galactosidase-specific T cells to provide help for an anti-hapten response

utilizing FITC as the hapten (103). The T cells used in this study were derived from animals primed to either the intact galactosidase molecule or various cyanogen bromide peptides derived from the enzyme. While T cell proliferation assays demonstrated that a high degree of priming was obtained to 6 cyanogen bromide peptides and an intermediate degree of priming to another 4 peptides, with only 2 of these 10 cyanogen bromide peptides did they demonstrate significant help for a FITC-galactosidase antibody response. Thus, most of the proliferating T cells specific for cyanogen bromide peptides did not provide efficient help. Unfortunately no information was provided as to whether the FITC was conjugated within the galactosidase molecule and whether there was a relationship between the site of conjugation and the peptides that provided help. But the selectivity of help in this system is consistent with the data and conclusions of Manca *et al.* described above.

These two studies, together with the observation of Berzofsky, are highly suggestive of a relationship between the antigenic determinant recognized by the immunoglobulin produced by the B cell on the one hand, and the epitopes that the B cell generates via processing for T cell recognition and T/B interaction on the other hand. Clearly, a great deal more work must be done to more precisely define the relationship between these different epitopes and the mechanism by which T cell specificity and B cell specificity are linked in these systems but the data are sufficiently suggestive to warrant a major effort in this area.

To summarize, in this section several experimental systems have been described, the results from which were interpreted as providing evidence for defects in the capacity of antigen presenting B cells to provide an adequate stimulus to T cells. It was concluded that B cells may be (1) defective in providing antigen nonspecific costimulatory signals to T cells, (2) defective in their capacity to process antigen or present it in the context of Ia molecules, (3) defective stimulatory cells because they make Ia antigens distinct from non-B cells, and (4) restricted in their capacity to process distinct antigenic determinants depending on the epitopes recognized by their membrane Ig.

In some instances the data were very suggestive because the readout of T cell stimulation only required antigen/MHC recognition (e.g., IL-2 production by T cell hybrids). In other systems, the readout was far removed from the assumed antigen presentation event by B cells (e.g., antibody production) making it difficult to establish that the defect was at the level of antigen presentation. Ultimately it will be necessary to determine the precise nature of processed antigen and the requirements for T cell triggering before a meaningful comparison of the capacity of B and non-B APC can be made.

VII. Concluding Remarks

It is clear that B cells share with other MHC class II-expressing cells the capacity to present antigen to T cells. The unique characteristic of B cell antigen-presenting capacity lies in the ability of specific B cells to efficiently take up antigen via their immunoglobulin receptors and to present that antigen to antigen-specific helper T cells. It has recently been established that the presentation of antigen by antigen-specific B cells is highly efficient compared to non-Ig-mediated antigen presentation by B cells or non-B cells due to the fact that these latter cells do not have the requisite antigen-specific receptors to facilitate antigen uptake. Although this proposed mechanism of antigen presentation to T cells by B cells explains many features of T–B interactions, such as the MHC restriction and the requirement for hapten and carrier to be on the same macromolecule in order to facilitate efficient T–B interaction, the relevancy of this mechanism for an *in vivo* immune response remains to be demonstrated. Furthermore, several aspects of the mechanism underlying this presentation still are not clear. Among these are the events involved in antigen processing. In particular where in the cell antigen processing takes place and how the relevant epitopes become expressed on the surface of the antigen-presenting cell remain undefined.

The question of the state of activation of B cells and T cells that permits successful presentation of antigen by B cells still has to be determined. For instance, can B cells trigger antigen-specific resting T cells or do the T cells have to have been recently stimulated by antigen-presenting non-B accessory cells before they can effectively interact with antigen-presenting B cells? In addition are other molecules besides Ia important for the accessory cell function of B cells and to what extent are these molecules expressed at different stages of B cell activation?

The relationship between the epitopes recognized by antigen-specific B cells and those that are recognized by the cooperating T cell is of considerable potential importance. Future studies will be needed to explore this relationship and in particular to analyze the spatial relationships between the T cell and B cell epitopes. Somewhat related to this, the epitopes generated by antigen processing and the signals delivered to T cells by B cells compared to non-B cells will require additional investigation.

The differential ability of B cells vs non-B accessory cells to produce costimulatory signals required for T cell activation and the nature of any costimulator molecules expressed by B cells compared to non-B accessory cells is currently controversial but should be resolved in the near future. Finally, the structure of Ia antigens on B cells and non-B cells as well as B

cells at different stages of activation need to be further explored with respect to the function of B cells as antigen-presenting cells for both conventional antigen as well as stimulatory cells in mixed lymphocyte reactions.

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ADVANCES IN IMMUNOLOGY, VOL. 39

Ligand–Receptor Dynamics and Signal Amplification in the Neutrophil

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I. Introduction

Leukocytes respond to a large number of stimulatory molecules which they encounter in the course of host defense and other inflammatory responses. These responses depend upon three distinct steps: (1) recognition of the stimulatory ligands by specific cell surface receptors, (2) the transduction of the binding event into an intracellular signal for cell activation, and (3) the ultimate activation of the metabolic pathways responsible for the appropriate cell functions.

Cell responses are coordinated and highly regulated. Since leukocytes are called upon to perform a variety of physiological roles ranging from chemotaxis to phagocytosis, their precise responses depend upon the conditions of stimulation: the type of stimulus, the rate of stimulus binding, the history of the previous cell responses, the type of surface with which the cells are in contact, and the presence of modulators which either amplify responses or diminish them. It is remarkable that ligands which are chemoattractants at very low concentrations require considerably higher ligand concentrations to elicit optimal free radical production and degranulation.

The goals of this article are to describe, insofar as possible at a molecular level, those pathways which regulate cell activation, and to elaborate a conceptual framework to account for the sensitivity of cell function to the conditions of stimulation. Our perspective will be quantitative to the extent that we will consider the (1) stoichiometry, (2) amplification, and (3) duration of receptor activation and signal generation. This approach relies to a significant extent upon dynamic and kinetic measurements. Second, we are taking a prospective view of leukocyte activation which places it into a context of other well-defined receptor systems (e.g., the photoreceptor and the β-adrenergic receptor) which contribute to the activation of other cell types. Finally, this article will be synthetic. At a number of points in the activation sequence we will employ preliminary data and we will speculate as to the quantitative relationships among the activation events.

TABLE I
NEUTROPHIL RECEPTORS

Receptors	Number (external)	Mass (on PMN) (kDa)	Function
Stimulatory			
Formyl peptide	<100,000	50–70[a]	Complete activator
C5a	<200,000	52[b] 48[c]	Complete activator
PAF[d]	<100,000	N.D.	Complete activator
LTB_4	~200,000	60[e]	Complete; oxidant production limited
CR1 (C3b)[f]	<10,000	205[g] 160–250[h]	Phagocytosis
CR3 (C3bi)[i]	<20,000	180[j] (two chains)	Phagocytosis
Fc (IgG)	<500,000[k]	50[l] (variable)	Complete except chemotaxis
Inhibitory			
β-Adrenergic	1,000[m]	N.D. PMN 45–65[n] elsewhere	Elevates cAMP Inhibits activity
PGE_1[o]	200 (monocyte)[p]	?	Elevates cAMP Inhibits activity
Adenosine A2[q]	10,000	?	Elevates cAMP Inhibits activity
Histamine H2[r]	?	40 (lymphocytes)	Elevates cAMP Inhibits activity

[a] Niedel *et al.* (1980); Niedel (1981); Dolmatch and Neidel (1983); Painter *et al.* (1982); Schmitt *et al.* (1983); Hoyle and Freer (1984); Marasco *et al.* (1985b).
[b] Rollins and Springer (1985); Johnson and Chenoweth (1985).
[c] Huey and Hugli (1985).
[d] Benveniste and Arnoux (1983).
[e] Goldman *et al.* (1985a).
[f] Unkeless and Wright (1984); Berger *et al.* (1984, 1985); Berger and Cross (1984); Todd *et al.* (1984).
[g] Fearon (1980).
[h] Dykman *et al.* (1983).
[i] Unkeless and Wright (1984); Berger *et al.* (1985); O'Shea *et al.* (1984).
[j] Beller *et al.* (1982); Wright *et al.* (1983a,b).
[k] Alexander *et al.* (1979).
[l] Unkeless and Wright (1984).
[m] Galant and Britt (1984).
[n] Lefkowitz (1982); Smigel *et al.* (1984).
[o] Lad *et al.* (1985a).
[p] Eriksen *et al.* (1985).
[q] Cronstein *et al.* (1985); Iannone *et al.* (1985).
[r] Beer *et al.* (1984).

II. Ligand–Receptor Dynamics

A. Types of Stimuli

Stimulatory ligands for PMN may be classified according to the functions they elicit (e.g., chemotaxis, phagocytosis, free radical generation, adherence, etc.), the form in which they are present (e.g., soluble, insoluble, and particulate; monovalent and multivalent, etc.), or their source. A number of physiologically relevant stimulatory and inhibitory ligands for PMN are listed in Table I, along with the reported number of cell surface receptors, characterization of the receptors, and cell functions associated with receptor occupancy by activating ligands.

Quantitative studies of ligand–receptor interactions have been accomplished most successfully with high-affinity monovalent ligands which can be appropriately labeled. This category includes at least four ligands: C5a FMLP[1] (and several of its analogs), LTB_4, and PAF.

The binding, dissociation, and internalization of formyl peptide and its receptor have been widely studied. Since rate constants are available for these processes, the interaction of this ligand and its receptor will serve as a general model for PMN ligand–receptor interactions (see below). Extensive characterization of the specific binding of C5a to its receptors has been accomplished (Chenoweth and Hugli, 1978, 1980; Chenoweth, 1981; Huey and Hugli, 1985) and rate parameters for C5a receptor dynamics are beginning to become available.

LTB_4 receptors were demonstrated by Goldman and Goetzl (1982) and Kreisle and Parker (1983). Goldman and Goetzl (1984) identified two populations of sites (4×10^3 high affinity, $K_d = 3.9 \times 10^{-10}$ M; 2.7×10^5 low affinity, $K_d = 6 \times 10^{-8}$ M) but rate parameters have yet to be established.

[1] Abbreviations: attomole, 10^{-18} mol; DAG, diacylglycerol; FLPEP, *N*-formyl norleucyl leucyl phenyalanyl norleucyl tyrosyl lysine-fluorescein; FMLP, *N*-formyl methionyl leucyl phenylalanine; FNLP, *N*-formyl norleucyl leucyl phenylalanine; G proteins, GTP binding proteins associated with cellular transduction; G_i, the G protein associated with inhibition of adenylate cyclase; G_0, a coupling protein derived from brain; G_p, the pertussis toxin-sensitive neutrophil G protein with mass similar to G_i; G_s, the G protein associated with the stimulation of adenylate cyclase; G*, the active state of the G protein, generally thought to be the α subunit bound to GTP; IP, inositol-1-phosphate; IP_2, inositol-1,4-bisphosphate; IP_3, inositol-1,4,5-trisphosphate; LR, a ligand–receptor complex, generally used here to denote the association of a formyl hexapeptide (FLPEP) with the formyl peptide receptor; LR*, the active form of LR; LR_0, the inactive form of LR; LTB_4, leukotriene B_4; PAF, platelet activating factor; PA, phosphatidic acid; PdBu, phorbol dibutyrate; PI, phosphatidylinositol; PIP, phosphatidylinositol-4-phosphate; PIP_2, phosphatidylinositol-4-5-bisphosphate; PKC, protein kinase C, a ubiquitous kinase dependent upon Ca^{2+}, phospholipid, and DAG; PLC, phospholipase C, a lipase which cleaves the phosphodiester bond converting phospholipid into DAG and a soluble headgroup; PMA, phorbol myristate acetate; PS, phosphatidylserine.

This situation may be improved using the 20-OH form of LTB_4 which binds to LTB_4 receptors with greater specificity than LTB_4 itself (Clancy *et al.*, 1984). PAF receptors exhibit a $K_d = 5 \times 10^{-9}$ *M* (Hwang *et al.*, 1983). Only preliminary dynamic information is available (Shen *et al.*, 1985).

It is worth noting that studies of the binding of multivalent ligands (immune complexes or C3b coated particles) are complicated by the difficulty in assessing the number of receptors actually engaged in binding individual particles as well as the time course over which contacts are made between individual receptor molecules and individual ligands on a single particle.

B. The Relationship of Ligand–Receptor Interaction and Cell Activation: The Static Model

Studies of the apparent equilibrium between formyl peptides and their receptors have been performed by several investigators in an effort to relate the extent of receptor occupancy to the magnitude of cell function. Often the binding studies have been performed at 4°C to eliminate the contribution of internalization and the attendant difficulties in identifying irreversibly bound ligand. Thirty minutes is regarded as adequate to achieve equilibrium.

Following early studies by Schiffmann and co-workers (1975) which identified chemoattractant activities of formyl peptides and identified the receptors through radioligand binding studies (Aswanikumar *et al.*, 1977; Williams *et al.*, 1977), Zigmond and co-workers (1979, 1982) initiated kinetic studies on ligand binding to rabbit peritoneal neutrophils which were adherent to petri dishes. The ligand was tritiated FNLP. From an analysis of the kinetics of ligand binding and dissociation at 4°C they reported that the equilibrium binding constant was consistent with the value expected based upon the on and off rate constants. They found that ligand binding could be approximated by a single class of binding sites. However, ligand binding was not strictly reversible to the extent that the quantity of dissociable ligand decreased as the length of the binding period increased.

A large number of investigators (Niedel *et al.*, 1979; Fletcher *et al.*, 1982; Sklar and Finney, 1982; Lohr and Snyderman, 1982; Yuli *et al.*, 1982; Mackin *et al.*, 1982; Koo *et al.*, 1983; Sklar *et al.*, 1984; Marasco *et al.*, 1983, 1985a,b), have used equilibrium or kinetic procedures to characterize ligand binding at the formyl peptide receptor. In some cases computerized Scatchard type analyses were used to fit the binding data. For example Mackin *et al.* (1982) reported two classes of sites, while Yuli *et al.* (1982) found a single class in sites in cells. In isolated membranes, investigators found two classes of sites for FMLP, a smaller fraction of high-affinity sites (20–30%, K_d approximately 1 n*M*) and a large fraction of low-affinity sites (K_d approximately 20–60 n*M*) (Koo *et al.*, 1983; Lane and Snyderman, 1984;

Marasco *et al.*, 1985). Koo *et al.* (1983) and Lane and Snyderman (1984) have also reported that the relative proportion of these classes of sites was sensitive to GTP. Kinetic investigations by Koo *et al.* (1983) demonstrated that guanyl nucleotides increase the net dissociation rate constant for formyl peptides by increasing the proportion of low-affinity sites. In these analyses, only 10% of the total membrane sites (approximately 50% of the highest affinity sites) was influenced by nucleotide. Zigmond *et al.* (1985) have reported that affinity of the receptor for ligand was reduced by Na^+, much like other receptors which are coupled to GTP binding proteins. The interactions between neutrophil receptors and GTP binding proteins will be discussed separately in Section III.

Snyderman and colleagues (1983; Lohr and Snyderman, 1982; Yuli *et al.*, 1982) have also investigated the impact of pharmacological modulators on the affinity of the receptor and on subsequent cell responses. They found that aliphatic alcohols which are membrane fluidizing agents increase receptor affinity at 4°C and lead to increased expression of chemotaxis and decreased expression of oxidant production and lysozomal enzyme release at 37°C (Yuli *et al.*, 1982). In contrast, the polyene antibiotics amphotericin B and nystatin which complex cholesterol reduce formyl peptide binding to its receptor at 4°C and reduce chemotaxis at 37°C (Lohr and Snyderman, 1982). These observations have led to the proposal that high-affinity sites transduce chemotaxis while low-affinity sites transduce oxidant production and degranulation (Snyderman, 1983; Snyderman *et al.*, 1983).

Goldman and Goetzl (1984), reporting heterogeneity of LTB_4 receptors on human neutrophils, also fit their binding data in terms of high- and low-affinity binding sites. They suggested that based on the affinity of the binding sites compared to the ED_{50} for chemotaxis that the high-affinity sites were responsible for chemotaxis. The ED_{50} for chemotaxis fell between the affinities of the two classes of sites.

Binding studies performed at 4°C could give an incomplete picture of the relationship between ligand–receptor interaction and cell activation for several reasons. The low temperature binding studies yield an essentially static picture of low- and high-affinity sites. While the sites may be converted by pharmacological agents or may be influenced by GTP, the rates and magnitudes of these processes at 4°C, or their relevance to cell activation at 37°C is not clearcut.

C. Ligand–Receptor Dynamics at 4°C

The results of Zigmond *et al.* (1979, 1982) and Sklar *et al.* (1984) which indicate a time-dependent slowly dissociable or even irreversible component of ligand binding at 4°C on cells need to be reconciled with the interpretation of equilibrium binding of the other workers. In the strictest

sense, it is inappropriate to analyze binding data which are not strictly reversible with a Scatchard type approach. The problems associated with Scatchard analysis of nonequilibrium interactions are considered widely in the pharmacology literature (DeLean and Rodbard, 1979; Ketelsegers *et al.*, 1984).

For these reasons we and others have examined ligand–receptor interactions in a dynamic or kinetic sense (Sklar and Finney, 1982; Marasco *et al.*, 1983; Sklar *et al.*, 1984). Our measurements have taken advantage of fluorescence procedures which have permitted real-time and, in some cases continuous, observation of ligand–receptor dynamics. The prime advantages of these spectroscopic techniques are that (1) they do not require separation or wash steps whose duration would obscure a rapid loss of ligand which could be associated with a low-affinity or rapidly dissociating state (particularly at 37°C), and (2) since these methods can permit continuous observation, the time resolution is on the order of 1 second.

As a ligand we used the fluorescein conjugate of the hexapeptide *N*-formyl-nLeu-Leu-Phe-nLeu-Tyr-Lys originally synthesized and characterized by Niedel (Niedel *et al.*, 1979). At 4°C in whole cells we observed that the ligand dissociability was lost as a function of time, and that at least 2 hours was required for ligand binding to approach equilibrium (Sklar *et al.*, 1984). After this period of binding we found the ligand to reside in a slowly dissociating state which has a half-time of dissociation of at least 30 minutes.

This slowly dissociating state was also observed in competitive binding studies (Sklar *et al.*, 1985c). Several nonfluorescent ligands including the hexapeptide and the tripeptide *N*-formyl-nLeu-Leu-Phe were preequilibrated with cells and then displaced by the fluorescent ligand. The rate and extent of displacement, both functions of the rate at which the prebound ligand dissociates from the receptor, were found to be a function of the time during which the ligand was preequilibrated. This slowly dissociating state was demonstrated not to arise from internalized ligand (Sklar *et al.*, 1984).

There are at least two possible explanations for the loss of dissociability of ligand at 4°C which have not yet been resolved. The loss of ligand dissociability in cells could reflect a G protein interaction parallel to that observed in membranes by Koo *et al.* (1983) and analogous to that detected in other cell surface receptors such as β-adrenergic receptors which are coupled to G_s and adenylate cyclase. Thus, the loss of rapidly dissociating ligand could reflect a time-dependent alteration in receptor interaction with a particular GTP binding protein, in this case G_p (see Section III).

A second possibility which could account for the time-dependent loss of ligand dissociability (which occurs rapidly at 37°C) is the interaction of the ligand–receptor complex with the cytoskeleton as reported by Jesaitis and co-workers (1984). These investigators were able to isolate a "high-affinity"

tertiary complex of ligand, receptor, and cytoskeletal elements. With the tripeptide *N*-formyl-Met-Leu-Phe, however, Jesaitis *et al.* (1984) found only a minimal association of this complex with the cytoskeleton for the first 20 minutes of ligand binding at 4°C. It has not yet been determined whether this limited cytoskeletal association is because insufficient time was allowed for it to form. However, this observation emphasizes the fact that binding studies performed at 4°C may not accurately reflect more rapid ligand processing which occurs at 37°C. It is also possible that the rate of formation of this slowly dissociating form is dependent upon the specific ligand so that it may occur slower for the radioactive tripeptide which Jesaitis *et al.* (1984) used compared to the fluorescent hexapeptide examined by Sklar *et al.* (1984, 1985a,c).

This loss of dissociability at 4°C appears to occur only with ligands which are full agonists for the receptor, as competitive binding studies have shown that it does not occur with the antagonists tboc-Phe-Leu-Phe-Leu-Phe and cbz-Phe-Met at least for periods up to an hour nor with the partial agonist f-Met-Phe (Sklar *et al.*, 1985c). This result may be analogous to the observation that stimulatory ligands induce down regulation of the receptor (Vitkauskas *et al.*, 1980).

D. Ligand–Receptor Dynamics at Intermediate Temperatures

The examination of ligand–receptor interactions at intermediate temperatures (15 to 25°C) permits the resolution of overlapping and rapid events (Marasco *et al.*, 1983; Jesaitis *et al.*, 1984; Sklar *et al.*, 1984; Anderson and Niedel, 1984). Kinetic measurements at 37°C are complicated by rapid ligand processing to intracellular compartments which appear to be preceded by even more rapid reorganization at the cell surface prior to internalization. The reduced temperatures offer a possibility of differentially influencing the various events and thus studying them selectively.

At 15°C, loss of ligand–receptor complex via internalization is considerably more rapid than at 4°C, but nonetheless proceeds with an apparent half-time in excess of 3 hours (Sklar *et al.*, 1984). However, Jesaitis *et al.* (1984) found a much more rapid formation of cytoskeletal complex with a half-time of approximately 20–30 minutes. The fluorescent ligand showed a conversion from a rapidly dissociating state (half-time for dissociation of approximately 2 minutes) to a slowly dissociating state (half-time for dissociation approximately 30 minutes) with a half-time for conversion of approximately 30 minutes. The loss of dissociability follows a similar time course to the formation of the cytoskeletal complex, but it has not been determined whether the slowly dissociating state formed prior to or as a consequence of cytoskeletal association.

The loss of dissociability proceeds with a half-time of about 3 minutes at

25°C but is complicated by the onset of rapid internalization. Taken together, all of these observations point to the fact that a description of ligand binding must take into account converting receptor states rather than a static population of high- and low-affinity receptors coupled to different cell responses. In order to attempt to relate the significance of these interconverting states to cell function, we must (1) discuss the dependence of various cell responses on receptor occupancy at 37°C, and (2) analyze high-resolution measurements of ligand–receptor dynamics at 37°C under conditions which parallel cell activation.

E. Transient Responses and Occupancy-Response Relationships at 37°C

The classical approach to relating ligand binding and cell response is to compare the K_d of the ligand to the ED_{50} for cell responses. Measurements of this type have been performed on several dozen ligands synthesized by Freer and Becker and colleagues (Showell *et al.*, 1976; Freer *et al.*, 1980, 1982). They observed a correlation between the efficacy of the ligands with respect to two functions (chemotaxis and lysozomal enzyme release) and the K_d for ligand binding. The ED_{50} for chemotaxis is typically 10-fold lower than for degranulation while the ED_{50} for degranulation and the K_d are comparable. Since cell responses at 37°C are elicited rapidly and prior to the equilibration of the ligand, the endpoint or static binding analyses at 4°C do not, however, unambiguously define the number of receptors which actually contribute to particular cell functions. If there were two distinct classes of receptor, the low ED_{50} values for chemotaxis could be consistent with the contribution of a high-affinity (i.e., low K_d), low-capacity receptor subclass playing a role in chemotaxis with the low-affinity, high-capacity receptor subclass playing a role in secretory responses and oxidant production. If the receptor classes were interconverting, this conclusion would have to be reconsidered.

The potential interconvertibility of the receptors, its regulation, and its role in cell activation need to be addressed by dynamic approaches. A rationale of this approach is that if either the number of receptors occupied or the rate of receptor occupancy could be controlled during the course of cellular responses, it should be possible to determine how many receptors contributed to various cell responses (Sklar *et al.*, 1981a, 1982b, 1985a; Korchak *et al.*, 1984c; deTogni *et al.*, 1985a,b). It was anticipated that functions that were dependent upon particular signals would (1) require similar numbers of receptors, (2) vary in parallel when the rate of ligand binding was varied, and (3) decay in parallel when ligand binding to the receptors was interrupted.

The number of receptors occupied has been varied in a "pulse" protocol

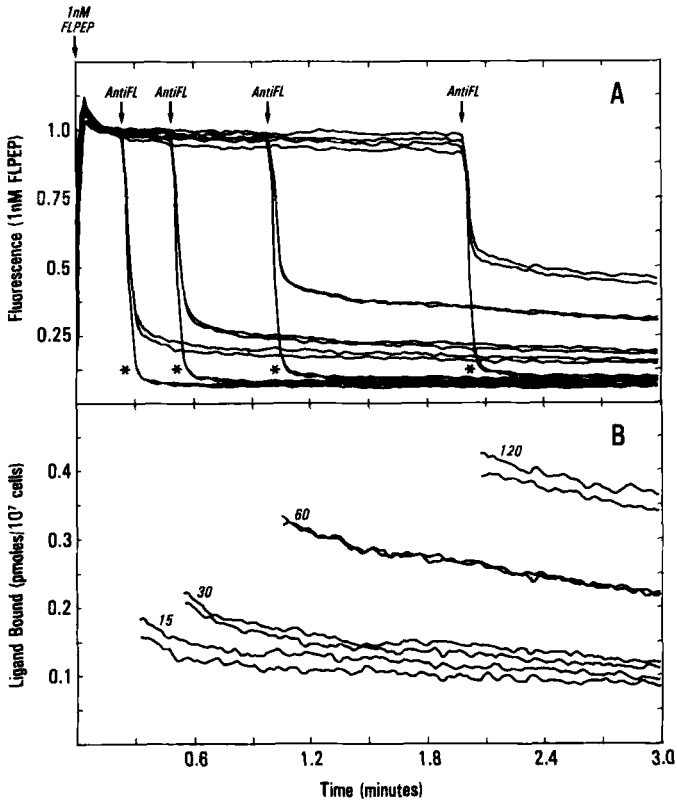

FIG. 1. Binding and dissociation of FLPEP under pulse conditions. (A) The data are plotted as FLPEP fluorescence versus time. At time 0, a cell suspension (10^7 PMN/ml) was exposed to 1 n*M* FLPEP. After 15, 30, 60, or 120 seconds antibody to fluorescein (25 n*M*) was added to bind and quench free ligand. The curves represent the amount of receptor bound FLPEP at the time of antibody addition and the dissociation of the ligand from the receptor thereafter. The curves with * represent background fluorescence in the presence of 10 μ*M* tBoc-Phe-Leu-Phe-Leu-Phe. (B) The specific binding and dissociation from (A). (Reprinted with permission, *J. Biol. Chem.* **260**, 11461–11467, 1985.)

(Sklar *et al.*, 1981a, 1982b, 1985a; Korchak *et al.*, 1984c), performed in either of two ways, one of which is illustrated in Fig. 1. The pulse is accomplished by initiating cell stimulation with an injection of the ligand. The number of receptors occupied is controlled by the concentration of the stimulus (the "height of the pulse") and the length of the period of ligand binding (the "width of the pulse"). The length of the period of binding of the ligand can be controlled either by the use of a receptor antagonist or an antibody to the ligand.

The antagonist functions by filling the receptor sites and limiting further stimulus binding. The antagonist must be administered at concentrations which are large enough to saturate the available receptors rapidly, in a period of seconds. Through the use of competitive binding studies and analysis of functional inhibition, Sklar *et al.* (1985c) established that the antagonist tboc-Phe-Leu-Phe-Leu-Phe to the formyl peptide receptor filled the available receptor sites in about a second when its concentration was >1 μM. The antibody approach is predicated upon interfering with the unbound stimulus in solution. Sklar *et al.* (1981a) used fluorescein isothiocyanate as a hapten conjugated to the formyl hexapeptide, *N*-formyl-nLeu-Leu-Phe-nLeu-Tyr-Lys. Antibodies to fluorescein bind the fluoresceinated peptide in solution and interfere with the binding of the peptide to its receptor. The antibody–hapten interaction occurs in solution with nearly diffusion-limited kinetics and is completed with a half-time of less than a second even at nM concentration. At the time of this review, interruption of cell stimulation by a pulse approach has been reported for formyl peptide and for concanavalin A by α-methylmannoside (Cohen *et al.*, 1982). In principle, however, antihapten antibodies for stimuli like fluoresceinated C5a or LTB_4 could be used. Antagonists to other receptors, such as kadsurenone for the PAF receptor (Shen *et al.*, 1985) could be applied. A comparison of the effects of dynamic response regulation at different receptors should prove valuable.

Several fundamental types of observations have been produced by this pulse procedure. Responses can be classified according to (1) the relationship of the time course of ligand binding to the time course of the response, (2) the behavior of the response once the ligand binding is interrupted, and (3) the number of receptors required to elicit an "optimal" response based either on its magnitude or duration. The first characterization is a semiquantitative description used by Sklar *et al.* (1981a, 1982b) and Korchak *et al.* (1984c). In the earliest studies, it was shown that several responses depended differentially upon binding. The "late responses," superoxide generation and cell aggregation, depended upon more than a minute of ligand binding. Several "early responses," degranulation (in cytochalasin B-treated cells), membrane depolarization, Ca^{2+} elevation and fluxes, and light scattering, could all be elicited by the ligand binding which occurred within seconds of exposure to the stimulus. Preliminary estimates suggested that a few percent of the receptors was required for the early responses.

Subsequently, more detailed studies have uncovered subtlety in the regulation of the responses (Sklar *et al.*, 1985a). Maximal (in magnitude) actin polymerization and transient right angle light scatter require less than 1% of the receptors; maximal Ca^{2+}, cAMP, depolarization, and degranulation require less than 10%; maximal oxidant production and aggregation require up to 100% of the receptors (Fig. 2). However, the magnitude of the response is

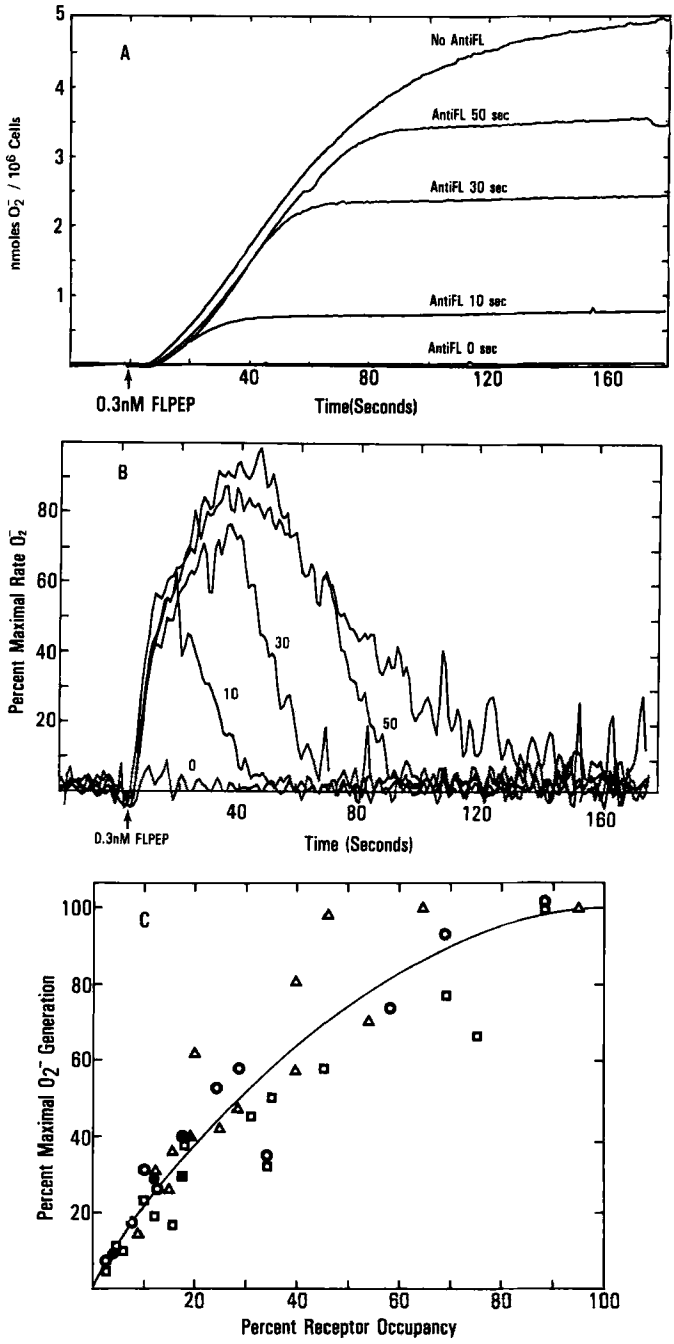

FIG. 2. Pulse response analysis of free radical production. (A) A fluorescent assay is used to measure the oxidant production of neutrophils stimulated by 0.3 n*M* FLEP. Antibody to fluorescein was added at 0, 10, 30, 50 seconds or not at all. (B) Rate of oxidant production, calculated as the first derivative from (A). (C) Occupancy–response curve based on pulse stimulation. For details, see *J. Biol. Chem.* **260,** 11461–11467, 1985. Reprinted with permission.

an ambiguous characterization. That is, for at least five responses (light scatter, actin polymerization, Ca^{2+}, oxidant production, aggregation in the absence of cytochalasin B), the responses decay to basal values shortly after ligand binding is interrupted. Unless ligand binding is allowed to continue at its normal rate these responses can achieve maximal levels, but the responses are not sustained at those levels. (For superoxide anion generation a maximal rate of production is observed when an estimated 10–20% of the receptors are rapidly occupied, but the rate of superoxide generation falls to zero when binding is halted.)

These observations imply that cell responses are transient unless sustained by ligand binding. Do the responses require continued occupancy of receptors already occupied or occupancy of new receptors? Korchak *et al.* (1984c) suggest that commitment to cell responses depended upon continuous occupancy. They found that in cytochalasin B-treated cells, receptors appear to be dissociated in a time period similar to the decay of the cell responses. By contrast, in the nontreated cells we found that the receptors were occupied and remained on the cell surface long after the period in which the cell responses decayed (Sklar *et al.*, 1984, 1985a). The responses decayed with a half-time of roughly 15 seconds (with the exception of cell aggregation which began to decay only after the cell cytoskeleton had relaxed). In contrast, at 37°C, bulk ligand dissociation occurs with a half-time of 2–3 minutes (Fig. 1 and Sklar *et al.*, 1984, 1985c).

Why do different cell responses depend to varying degrees upon receptor occupancy? The explanation (Sections IV and V) relates branchpoints in the activation sequence to the idea that the biochemistry of individual response pathways can be saturated by different numbers of receptors. The relevance of transient responses to ligand–receptor dynamics is considered below.

F. A Model for Ligand–Receptor Dynamics

The simplest possible model for short-term ligand–receptor dynamics for the fluorescent hexapeptide is shown below:

$$L + R \underset{0.3/\text{minute}}{\overset{10^9\ M/\text{minute}}{\rightleftharpoons}} LR \xrightarrow{0.2/\text{minute}} LR_{int}$$

This simple model for the fluorescent hexapeptide indicates that the ligand and receptor interact reversibly but that the ligand–receptor complex can either dissociate or be internalized, each process occurring with a half time of a few minutes. For the tripeptide FNLP, the on-rate constant differs but the rates of dissociation and internalization are comparable (Zigmond *et al.*, 1982). This simple model appears to be inadequate to account for much of the experimental observations cited above and provides little insight into cell activation for the following reasons.

First, low temperature equilibrium binding measurements suggest the likelihood of several classes of receptor affinities which are modulatable by GTP, alcohols, and ions.

Second, kinetic measurements at low and intermediate temperatures demonstrate a time-dependent loss of dissociability of ligand which occurs while the ligand–receptor complex is on the cell surface prior to internalization.

Third, the loss of dissociability is roughly paralleled in time by an attachment of the ligand–receptor complex to the cytoskeleton.

Fourth, the rate constant (0.3/minute) which describes the bulk dissociation of the ligand–receptor complex at 37°C characterizes a state of the receptor which appears to be associated with no cell activation. That is, following a pulse of cell stimulation (Fig. 2), one finds cell responses having decayed with a half-time of 15 seconds following the inhibition of ligand binding, yet most of the ligand remains bound to the cell surface receptors at the time that the cell responses have ceased (Fig. 1). These occupied receptors are producing no cell response at this time.

Rossi *et al.* (1983) observed that the rate of oxidant production was not proportional to the number of receptors occupied and proposed that the activity of ligand–receptor complexes was short-lived. Sklar *et al.* (1985a) suggested that the lifetime of the active receptor must be shorter than the total period for the responses to decay (30–40 seconds). We hypothesized that the lifetime of the active receptor might actually be limited to the period between which ligand binding was interrupted and the cell responses began to decay (5 to 10 seconds).

Sklar (1985) proposed that high-resolution formyl peptide receptor dynamics at 37°C could be accounted for by a model which permits the receptor to exist in rapidly interconverting active and inactive states.

$$\begin{array}{l} \mathrm{L + R} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{LR^*} \overset{k_2}{\rightarrow} \mathrm{LR_0} \overset{k_3}{\rightarrow} \mathrm{LR_{int}} \\ \qquad\qquad\qquad\quad \underset{k_{-2}}{\downarrow} \quad \overset{k_3}{} \\ \qquad\qquad\qquad\quad \mathrm{L + R_0} \rightarrow \mathrm{R_{int}} \end{array}$$

In this model the ligand and receptor combine to form a transiently active complex (LR*) which can either dissociate to the initial state or be converted into the inactive ligand–receptor complex (LR_0). Competing processes internalize this complex with a half-time of several minutes, or allow it to dissociate. The dissociation from this inactive state LR_0 is reflected in the bulk dissociation measured in the binding studies (Fig. 1). The pathway which dissociates LR_0 into $L + R_0$ is intended to reflect the possible irreversible attachment of the receptor with the cytoskeleton (Jesaitis *et al.*, 1984) or down-regulation (Vitkauskas *et al.*, 1980) in an inactive form where it can be internalized whether occupied (LR_{int}) or not occupied (R_{int}). The relevance

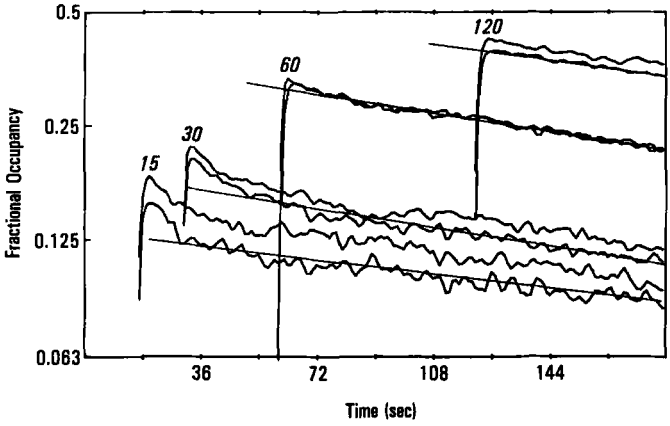

FIG. 3. Semilog plot of dissociation data from Fig. 1.

of such two state models has been considered by others (see Gero, 1983) and the need for such models in other receptor systems is apparent (e.g., insulin receptor, Donner and Corin, 1980; Lipkin *et al.*, 1986).

Data relevant to this model are provided in Fig. 3. The experiment examines the extent of ligand binding (1 n*M* fluoresceinated hexapeptide) to 10^7 cells/ml in suspension using fluorescence spectroscopy. Binding is interrupted after 15, 30, 60, or 120 seconds and dissociation is measured continuously. In separate experiments, internalization (0.2/minute) was measured. Of the five rate constants, the bulk on (k_1), off (k_{-2}), and internalization (k_3) rates come directly from binding, dissociation, and internalization data (simple model above; Sklar and Finney, 1982; Finney and Sklar, 1983; Sklar *et al.*, 1982a, 1984). The parameters describing the rapid dissociation of LR* (k_{-1}) and the conversion of LR* to LR_0 (k_2) are obtained by analyzing the high-resolution measurements for curvature in the dissociation immediately after binding is stopped. On a semilog plot, the bulk ligand dissociation following 2 minutes of binding is approximately linear (exponential with a half-time of 2–3 minutes). This reflects the dissociation of LR_0 into L + R_0 and is characterized by k_{-2}. At shorter periods of binding there is a rapid component of dissociation, with a half-time on the order of 10 seconds. This reflects the dissociation of LR* into L + R and is characterized by k_{-1}. The magnitude of this rapid dissociation diminishes rapidly as binding plateaus. In the model the loss of this rapid component with time is caused by the conversion of active LR* into inactive LR_0 (k_2).

The model makes a prediction about the lifetime of the active form of the receptor, LR* (Sklar, 1985). When binding is stopped, the active form of the

receptor decays with a half time of approximately 5 seconds. This period is comparable to the time it takes for cell responses to begin to decay once ligand binding has been interrupted (Fig. 2). The experimental results taken together with the model imply that cell responses and cell signals are sustained by the formation of active ligand–receptor complexes. Once these active complexes have disappeared, the cell responses begin to turn off.

Jesaitis (1985) has hypothesized that formation of the high-affinity or slowly dissociating form of the receptor that can be isolated in part on the cytoskeletal fraction of the cells may represent the turnoff step in transduction. The evidence supporting such an hypothesis rests on several observations. The first is that cytochalasins appear to inhibit formation of the slowly dissociating form of the complex on cells both at 37 and 14°C. The second is that at 14°C, a temperature at which internalization and degranulation in neutrophils are minimized, prior exposure to formyl peptide inactivates the cells by 90% to subsequent stimulation. This inactivation is stimulus specific and is not observed after subsequent stimulation with PMA. The third is that cell responses are elongated (i.e., do not shut down) in the presence of cytochalasins. Such results could be consistent with a role for the cytoskeleton in sequestering or inactivating the receptor in the plane of the membrane.

Another possible means of regulation has been recently obtained (Sklar *et al.*, 1986) using the permeabilized cell preparation described by Smolen *et al.* (1985, 1986) as shown in Fig. 4. Ligand binding in permeabilized cells appears to be characterized by a single class of sites with a uniform dissociation rate similar to LR_0 ($t_{1/2}$ approximately 2 minutes). Upon addition of guanine nucleotides, the receptors dissociate uniformly with a time constant similar to LR* ($t_{1/2}$ approximately 10 seconds). It appears that the presence of guanine nucleotide in permeabilized cells at 37°C can regulate the ligand–receptor interaction between states which parallel its active and inactive forms in functioning, intact cells at 37 °C.

The regulation of the lifetime of the active receptor in the intact cell remains poorly understood. It is not known whether an individual receptor may be activated repeatedly by multiple encounters with new ligand molecules or whether receptors are down-regulated by their first successful encounter. It appears that receptors which are dissociating slowly are no longer transducing signals and that such high-affinity receptors are associated with the cytoskeleton. However, it has not been established whether the loss of activity is due to the cytoskeletal attachment or if the recognition of the ligand–receptor complex by the cytoskeleton is a consequence of some chemical modification of the complex (phorphorylation?) which changes the receptor affinity and leads to its inability to couple to cell activation.

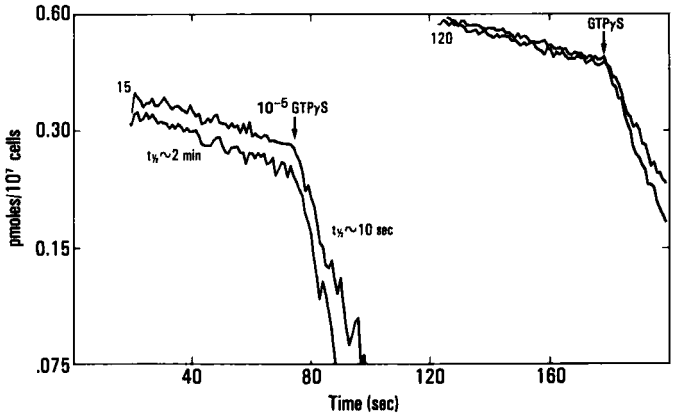

FIG. 4. Binding and dissociation in permeabilized cells. The data are plotted as the log of bound FLPEP versus time. Experimental conditions similar to Fig. 1, except that 10^7 permeabilized cell/ml were used. At time 0, 1 n*M* FLPEP was added to cell suspension. At 15 or 120 seconds antibody to fluorescein was added. At 75 or 180 seconds (respectively) 10^{-5} GTPγS was added.

III. Coupling Mechanisms

A simple scheme for the coupling of activated receptor to the generation of cell signals is provided in Fig. 5. This scheme involves (1) a transmembrane interaction of receptor with guanyl nucleotide binding proteins, (2) the activation of a catalytic species (most likely a phospholipid phosphodiesterase) by the nucleotide binding proteins, (3) the cleavage of phosphoinositides by the phospholipase, and (4) the generation of products from the phosphoinositides which lead to the elevation of intracellular Ca^{2+} and the translocation and activation of protein kinase C. While it is presumed that the activation of cell functions depends upon Ca^{2+}, calmodulin, and C kinase-dependent protein phosphorylation, there is not as yet a single neutrophil function whose sequence of activation can be traced in quantitative detail all the way from ligand binding to the receptor through the activation of the function producing entity.

In Sections III and IV we will consider the evidence in support of the scheme depicted in Fig. 5. We will consider the significance of branchpoints in the activation sequence, the existence of interacting stimulatory and inhibitory pathways, and some potential alternatives to the pathway depicted.

A. Potential Functions of Transiently Active Receptors

There are a variety of possible mechanisms by which liganded receptors transduce or initiate the transduction sequence required in cell activation.

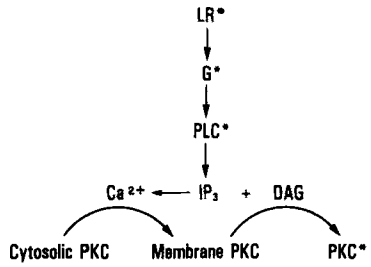

FIG. 5. Hypothesized sequence of activation events in neutrophil stimulation. LR*, Activated receptor; G*, activated G protein; PLC*, activated phospholipase; PKC*, activated protein kinase.

Three general classes of receptor activities have been indicated in different cell types. First, a receptor may possess its own activity as an enzyme (e.g., the epidermal growth factor receptor kinase, Buhrow *et al.*, 1982; Staros *et al.*, 1985, or the insulin receptor, Haring *et al.*, 1984), or as a channel (Hille, 1984) which is regulated by stimulus binding. Second, the receptor may interact directly with enzymes or channels, perhaps in a diffusion controlled manner, which produce the signals for cell activation. Third, receptors may interact with a specific class of coupling proteins which are intermediates between the activated receptors and the signaling systems (Stryer *et al.*, 1981; Hildebrandt and Birnbaumer, 1982; Smigel *et al.*, 1984; Stryer 1986). The preponderance of evidence is that neutrophil receptors associated with cell activation require, to a significant extent, the participation of coupling proteins.

While ligand binding to neutrophil cell surface receptors may evoke activities in the receptor or an interaction of the receptor with species other than coupling proteins, there is at present little direct evidence to suggest that these interactions participate in cell activation. Two types of interactions should be noted. The formation of the high-molecular-weight complex of formyl peptide receptors and cytoskeletal elements (Jesaitis *et al.*, 1984) is almost certainly too slow to be involved in the initiation of the cell activation sequence and is more likely to contribute to the regulation of cell function or ligand–receptor processing. It has also been observed that macrophage Fc receptors themselves function as ion channels which might lead to macrophage activation (Young *et al.*, 1983). These receptors could perform similar roles in neutrophil activation. However, evidence has been presented that signal transduction by Fc receptors is independent of ion flux (Pfefferkorn, 1984). The most compelling evidence for the means by which receptors transduce the stimulus binding event into an intracellular signal comes from studies designed to probe the roles of coupling proteins. More detailed information concerning the occurrence and biochemistry of the various fami-

lies of coupling proteins is provided in recent reviews (Smigel *et al.*, 1984; Stryer, 1986).

B. Coupling Protein Families

The coupling proteins consist of a family of guanyl nucleotide binding proteins. The coupling proteins from different sources consist of heterotrimers of $\alpha\beta\gamma$ subunits with a total mass of approximately 80 kDa. Information about the coupling proteins is derived primarily from two different receptor systems. The coupling protein G_s has been studied in systems involving the β-adrenergic receptor (Smigel *et al.*, 1984). The interaction of a stimulatory hormone (catecholamines such as epinephrine or isoproterenol) with its receptor leads to the activation of adenylate cyclase, an important regulatory enzyme for many cell types (Fig. 6). It is generally the case that systems which possess the "stimulatory" G_s also possess G_i, a coupling protein inhibitory to cell activation by G_s. Separate classes of "inhibitory" receptors, namely α-adrenergic receptors, lead to inhibition of adenylate cyclase.

The photoreceptor protein, rhodopsin, is coupled to the retinal G protein sometimes referred to as transducin (Stryer, 1986). Transducin activates a cGMP phosphodiesterase which appears to play a crucial role in visual transduction. A fourth G protein, G_0, is found in brain, but its role is not completely understood.

The action of G proteins as they are presently understood is that binding of a stimulatory hormone to its receptor induces dissociation of the α subunit (approximately 40–45 kDa) from the $\beta\gamma$ subunit of the particular G protein which interacts with the receptor (Fig. 6). The α subunits from the different G proteins possess guanyl nucleotide binding activity, GTPase activity, appear to be responsible for activation of the catalytic units (i.e., cyclase or phosphodiesterase), and possess regulatory sites which can modulate both interactions with the receptors and the catalytic units. While the α subunits are unique to each G protein species, the $\beta\gamma$ subunits appear to be identical and exchangeable.

The exchangeability of the $\beta\gamma$ subunits has lead to a proposal concerning the mode of G protein regulation, particularly between G_s and G_i (Smigel *et al.*, 1984; Gilman, 1984). It appears that hormone binding to receptors inhibitory to adenylate cyclase induce the release of $\beta\gamma$ subunits from G_i (Fig. 6). The $\beta\gamma$ subunits of G_i bind to the α subunit of G_s to reform intact G_s, and thereby regulate the cyclase activation. Since G_i is normally in excess over G_s, the production of excess $\beta\gamma$ subunit from G_i by inhibitory receptors can compete effectively for the α subunits of G_s and displace them from the cyclase.

It is of historical significance that as late as 1983, potential roles of G_i-like proteins as activators of cell function were uncertain.

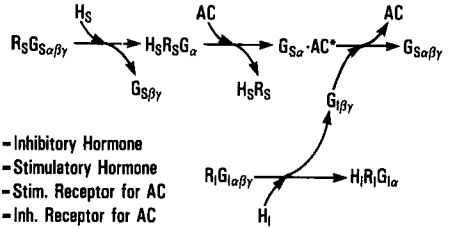

FIG. 6. Model for interaction between "stimulatory" and "inhibitory" G proteins. $G_{s\alpha\beta\gamma}$, "Stimulatory" heterotrimer, G_s; $G_{I\alpha\beta\gamma}$, "inhibitory" heterotrimer, G_I; AC, adenylate cyclase. In the scheme depicted, the stimulatory hormone H_s binds to its receptor R_s and causes the dissociation of $G_{s\alpha\beta\gamma}$ into $G_{s\alpha}$ and $G_{s\beta\gamma}$. $G_{s\alpha}$ is used in the activation of AC. An inhibitory hormone H_I binding to its receptor R_I causes the release of $G_{I\beta\gamma}$ from G_I. $G_{I\beta\gamma}$ is shown to displace $G_{s\alpha}$ from AC and to reform the heterotrimer $G_{s\alpha\beta\gamma}$.

C. Coupling Proteins in Neutrophils

The possible role of coupling proteins in the activation of neutrophils has been suspected for at least as long as it has been recognized that cAMP is elevated when the cells are stimulated. Probably the first direct evidence for G protein involvement comes from binding studies performed by Koo *et al.* (1983). They documented the fact that the binding affinity of formyl peptides to their receptors in neutrophil plasma membranes was reduced by the presence of guanyl nucleotides. This effect parallels what is observed when guanyl nucleotides bind to G_s and reduce the binding of catecholamines to β-adrenergic receptors. Shortly after these observations Hyslop *et al.* (1984) demonstrated that formyl peptide receptors in neutrophil homogenates were coupled to the activation of a GTPase. These observations were consistent with the possibility that the stimulatory neutrophil receptors were associated with the activation of adenylate cyclase and produced an elevation of cAMP when the cells were stimulated. Both of these observations (Koo *et al.*, 1983; Hyslop *et al.*, 1984) were made at a time when a possible stimulatory role for G_i-like proteins was not widely appreciated.

While these observations were consistent with a role for G_s, a hypothetical activation sequence involving G_s has long been questionable in neutrophils for several reasons. First, increasing cAMP levels in neutrophils by dibutyryl cAMP or by inhibitors of phosphodiesterases turns out, if anything, to be inhibitory to cell function elicited by formyl peptides receptors (Zurier *et al.*, 1974; Bourne *et al.*, 1971; Hills *et al.*, 1975; Simchowitz *et al.*, 1980a,b). In no case is there good evidence that such cAMP elevation is itself a stimulatory signal. Second, receptors which are classically coupled to cyclase activation have been recognized in the neutrophil to be at best nonstimulatory and, at worst, strongly inhibitory to cell activation (Busse and Sosman, 1984; Fantozzi *et al.*, 1984; Lad *et al.*, 1985a; Tecoma *et al.*,

1985; Nielsen *et al.*, 1985). Third, cholera toxin, which causes the activation of G_s and its uncoupling from its receptors, does not activate neutrophils (Bokoch and Gilman, 1984; Shefcyk *et al.*, 1985). Lastly, Simchowitz and colleagues (1983) produced evidence that cAMP elevation stimulated by formyl peptides could be pharmacologically obliterated without interfering with cell activation. Taken together, these results suggested strongly that neutrophil activation was not mediated through G_s.

If activation were not through G_s, then several puzzling results had to be accounted for: (1) cAMP is elevated during neutrophil activation, (2) GTPase activity is stimulated by ligand binding to the formyl peptide receptor, (3) guanine nucleotides influence ligand–receptor binding, (4) the likely second messengers in a neutrophil, Ca^{2+}, and metabolites of PI, need to come from somewhere, and (5) as appreciated later, receptors that couple to G_s are inhibitory to receptors that appear to couple to proteins similar to G_i.

The pieces of this puzzle have come together in the last few years in a neat and elegant package. It has been appreciated for a number of years that receptors which stimulate the metabolism of PI elevate intracellular Ca^{2+} (see reviews by Berridge, 1984; Marx, 1984; Majerus *et al.*, 1984). The potential roles of guanyl nucleotide regulatory proteins in these processes were exposed when Ui and colleagues (reviewed by Ui, 1984) recognized that the islet-activating protein or pertussis toxin was inhibitory both to receptor-mediated phosphoinositide turnover and Ca^{2+} elevation. Bokoch *et al.* (1983) obtained evidence that a substrate for pertussis toxin was, in fact, G_i, (and possibly G_0) the coupling protein known previously only for its inhibitory role in cyclase activation.

These observations led to the possibility that G_i (or a G_i-like protein) could have a general stimulatory function in certain cell types in addition to its better known inhibitory role. In a remarkably short period of time, Bokoch and Gilman (1984), Molski *et al.* (1984), Lad *et al.* (1985b–d), Shefcyk *et al.* (1985), Becker *et al.* (1985), Verghese *et al.* (1985a), Krause *et al.* (1985), Brandt *et al.* (1985), and Goldman *et al.* (1985c) were able to demonstrate that stimulatory receptors in neutrophils are coupled to cell activation via a coupling protein sensitive to pertussis toxin, "G_p." Bokoch and Gilman (1984) showed that arachidonic acid metabolism was sensitive to the extent of ADP ribosylation of a coupling protein subunit similar in molecular weight to the α subunit of G_i. To date, a number of responses including phosphoinositide turnover (Brandt *et al.*, 1985; Krause *et al.*, 1985), Ca^{2+} elevation (Molski *et al.*, 1985; Goldman *et al.*, 1985c), actin polymerization (Shefcyk *et al.*, 1985), free radical production, degranulation (Lad *et al.*, 1985c), chemotaxis (Becker *et al.*, 1985), aggregation, stimulus binding (Okajima *et al.*, 1985), and nucleotide exchange (Okajima *et al*, 1985) in the presence of stimulus have been shown to be sensitive to pertussis toxin. Observa-

tions of this sort include the stimuli LTB_4 and formyl peptides as well as PAF (Lad *et al.*, 1985b), C5a, and immune complexes (Lad *et al.*, 1985d), suggesting a common pathway for at least several aspects of the activation sequence.

A central role for G_p can only be rationalized if we can also account for the puzzling observations. First, the elevation of cAMP by formyl peptide receptors appears not to be a consequence of their interaction with G_s. Rather, it has been shown to be secondary to Ca^{2+} elevation. Jackowski and Sha'afi (1979) and more recently Verghese *et al.* (1985b) have shown that Ca^{2+} antagonists inhibit cAMP elevation. These results suggest that Ca^{2+} rises contribute to a transient inhibition of cAMP phosphodiesterase.

The ability of G_p to contribute to GTPase activity or guanyl nucleotide exchange is not unexpected. Yet it is probably fortuitous that stimulatable GTPase activity in crude neutrophil homogenates has been observed. This observation of GTPase activity may be possible only because G_i-like proteins are typically in great abundance compared to G_s and have a 10-fold greater intrinsic GTPase activity (Sunyer *et al.*, 1984). The fact that the maximally elevated GTPase activity occurs within approximately 10 seconds of ligand saturation of the receptors is consistent with this activity playing a regulatory role in cell activation. In one scheme of G protein action (Smigel *et al.*, 1984), ligand binding to the receptor induces dissociation of α and $\beta\gamma$ subunits from the G protein and exchange of GTP for GDP in the active complex of ligand, receptor, and α subunit of the G protein. Eventual cleavage of the GTP may permit inactivation of the G protein which then rebinds its own $\beta\gamma$ subunit to reform the resting state of the G protein (see Fig. 7).

The role of coupling proteins in the transduction sequence has been further substantiated by reconstitution studies in which neutrophils have been treated with pertussis toxin. When membranes have been isolated, the formyl peptide binding is reduced and formyl peptide fails to stimulate GTPase activity (Okajima *et al.*, 1985). Reconstitution of purified G_i restores ligand binding and ligand-dependent GTPase activity. A subsequent step, phosphoinositide turnover, can also be linked to receptor and G protein (Cockroft and Gomperts, 1985; Smith *et al.*, 1985; see below).

We have recently been able to examine some aspects of G protein–receptor behavior in a permeabilized cell system (Sklar *et al.*, 1986) at 37°C. Unlike what occurs in intact cells, the fluorescent formyl peptide does not undergo a time-dependent interconversion between rapidly and slowly dissociating forms in the absence of guanyl nucleotide (Fig. 4). In fact, all of the ligand is detected in a slowly dissociating form. At 37°C, the half-time for dissociation is 2–3 minutes for the slower form. This may be the form described as "LR_0." When the guanine nucleotide is added to the cell suspension, fluorescent hexapeptide immediately begins to dissociate from the receptor. For saturating guanyl nucleotide (e.g., 10^{-5} *M* GTPγS), the half-

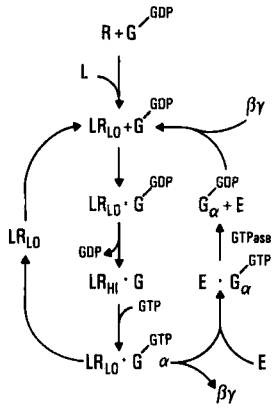

FIG. 7. Working model receptor–G protein interaction. Formation of LR results in rapidly dissociating low-affinity receptor, LR_{LO} (LR*?) which causes G to exchange GTP for GDP, G to dissociate into G_α and $G_{\beta\gamma}$, and LR to dissociate from G. G_α bound to GTP binds and activates transducing enzyme E (PLC) until GTP is hydrolyzed. LR_{LO} in principal, may recycle unless its lifetime is limited. G_α bound to GDP can add $\beta\gamma$ to reform intact G. LR–G in the absence of nucleotide is a slowly dissociating high affinity receptor. The extent to which LR_{LO} and G_α cycle and the rate of cycling are unknown.

time for ligand dissociation is about 10 seconds. We hypothesize that this state may correspond to LR*. By interpreting the binding model in terms of the permeabilized cell data, it appears that the state of the receptor governed by the G protein in the presence of nucleotide is the short-lived state of the receptor which corresponds to the active state of the receptor in the intact cell during the transduction process. The putative inactive state of the receptor may exist when nucleotide is absent. Two notable features of these results are apparent. All of the receptors in this preparation at 37°C can be trapped in distinct affinity states regulated by nucleotide. The interconversion between the nucleotide free state and the nucleotide bound state is within the time resolution of our present measurements, i.e., less than a second. The results have been suggested to mean that LR is associated with G when nucleotide is absent; the presence of nucleotide stimulates R–G dissociation (Fig. 7).

The final parts of the puzzle are discussed below. The production of intracellular messengers is discussed in Section IV. A possible role of cAMP and receptors which couple to G_s as inhibitors of receptors which are coupled to G_p is addressed in Section V.

D. Amplification at the Level of Signal Transduction

While the qualitative features of the role of G_p–receptor coupling are beginning to be established, almost none of the quantitative features has been defined. Given the possibility that the receptor stays active only for a

short time, the stoichiometry of receptor–G protein interaction becomes a crucial factor in regulating amplification during transduction. The ratio of stimulatory receptor to G_i in the neutrophil is not known with precision. Given the number of different receptor types, an estimate of up to 10^6 stimulatory receptor to G_p in the neutrophil is not known with precision. binding of guanyl nucleotides to neutrophil membranes, Bokoch (unpublished) estimates that there are a minimum of 3×10^5 guanyl nucleotide binding proteins per neutrophil in the plasma membrane. Since the abundance of G protein in other cell types rarely exceeds a few percent of the membrane protein (Sterweiss and Robishaw, 1984) with G_i-like, pertussis toxin-sensitive proteins in excess over G_s, we anticipate that G_p does not exceed several hundred thousand per cell. Since neutrophils are sensitive to both pertussis toxin and cholera toxin (which ADP ribosylate the α subunits of G_p and G_s, respectively) it is likely that both G_s and a G_i-like protein are present. However, G_0 appears to be absent by immunological criteria (Bokoch, unpublished). Definitive proof that the coupling protein associated with neutrophil stimulation G_p is indeed G_i or different from it remains to be established.

It is not possible as yet to ascertain definitively whether there is at least one, or more than one, G protein available per receptor in the neutrophil and to determine the extent of amplification. Different pictures for G protein–receptor amplification have emerged. For G_s it has been suggested that the α subunit of G_s remains attached to the activated β-adrenergic receptor, while model system studies suggest that up to 10 G_s molecules may interact per activated receptor in a sequential manner (Asano *et al.*, 1984). In contrast, a bleached rhodopsin (a photoreceptor molecule which has "bound" a photon) will interact with up to several hundred transducin molecules (Kuhn, 1984; Stryer, 1986).

In order to ascertain stoichiometry, reconstitution systems where receptor and G protein stoichiometry can be varied could prove useful. A measurement of the catalyzed binding of GTPγS by G protein per activated receptor should yield the desired information. Ultimately, more intimate details will be required. (1) Does the G protein become dissociated from the receptor either during or after the receptor activation? (2) Can a particular G protein interact with more than one receptor or more than one class of receptors? (3) Are all receptors or all receptor classes coupled to G proteins with equal probability?

E. Termination of G Protein–Receptor Interaction

Termination of this phase of transduction phase may in principle arise as a consequence of any of a number of factors. First, transduction may cease because receptor can no longer couple to G protein, either because receptor or G protein is lost, cleared, or modified. Second, transduction may cease

because G protein may no longer couple to the catalyst for the same reason. Reports (see Stryer, 1986, for details in photoreceptor systems) of both receptor modification (Lefkowitz, *et al.*, 1984) and G protein modification by phosphorylation (Katada *et al.*, 1985) are available for other cell systems. Termination of transduction is probably best characterized in the photoreceptors; two steps are involved (Stryer, 1986). Rhodopsin kinase phosphorylates activated rhodopsin which may limit rhodopsin–G protein interaction; moreover, a new protein called arrestin is also believed to compete with transducin for activated rhodopsin.

We anticipate that specific receptor modification and loss of coupling in the neutrophil occurs prior to receptor clearance because cell functions decay following pulse stimulation prior to the clearance of receptors. Whether G proteins can be cleared is unknown but this question may be answered with antibodies to G proteins.

F. Inhibitory Coupling Proteins

Receptors that are coupled through the pertussis toxin-sensitive G protein lead to PI turnover and Ca^{2+} elevation. Receptors that are normally coupled to G_s and the activation of adenylate cyclase (e.g., adrenergic receptors which bind catecholamines and receptors for prostaglandins) are inhibitory to neutrophil activation. The functional antagonism between these classes of receptors has been linked to the elevation of cAMP and will be discussed in more detail in Section V. It is remarkable that the inhibitory output of less than 10^3 adrenergic receptors can be coupled to the turnoff of the functional output of up to 10^5 formyl peptide receptors.

IV. Signals

The potential signaling events in cell activation may be roughly divided into those which are generated inside the cell in response to transmembrane transduction, or species which are permitted entry into the cell following transduction. The evidence presented above depicts transmembrane coupling of the occupied receptor with G proteins which are located on the cytoplasmic surface of the plasma membrane. This implies that a primary aspect of signaling involves cytoplasmic processes. It is well documented, nonetheless, that transmembrane ionic fluxes in neutrophils include Ca^{2+}, Na^+, K^+, and H^+ (Showell *et al.*, 1976, 1977; Korchak *et al.*, 1984a,b; Simchowitz, 1985a–c). The manner in which these fluxes are regulated and their integration into the activation process will not be addressed in this article.

This section will emphasize the likely relationships between the cytoplasmic signaling pathways. We will take the position that activated recep-

tors are coupled to the metabolism of phosphatidylinositol via the participation of G proteins and the activation of phospholipase C (PLC) (Fig. 5). The metabolites of various phosphoinositides include soluble inositolphosphates (IP, IP_2, IP_3), diacylglycerol (DAG), and several additional species which together lead to the elevation of intracellular Ca^{2+} and the activation of kinases including protein kinase C (PKC).

A. The Linkage between Coupling Proteins and the Turnover of Phosphatidylinositol

Evidence has recently become available which indicates that the activation of G_p is coupled to the activation of PLC. This putative pathway implies that activation of phospholipase C is not secondary to Ca^{2+} elevation, that Ca^{2+} elevation is a result of products of the action of PLC, and, therefore, the Ca^{2+} elevation during cell activation is not entirely a consequence of transmembrane Ca^{2+} flux. In addition, this suggests that G_p is coupled to PLC activation like other G proteins which couple to enzymes active on phosphodiester bonds (G_s/adenylate cyclase, transducin/cGMP phosphodiesterase, G_p/phospholipid phosphodiesterase = phospholipase C).

Several lines of evidence link G proteins to phosphoinositide metabolism. The effects of pertussis toxin indicate that when transmembrane transduction is blocked, phosphoinositide turnover, Ca^{2+} elevation, and other aspects of activation are inhibited (e.g., Krause *et al.*, 1985). These experiments alone do not imply a precursor/product relationship among any of the steps of activation. Indeed, kinetic studies by Cockroft *et al.* (1980) suggested that phosphoinositide turnover was slower than degranulation (and more recently it has been realized that phosphoinositide turnover is "slower" than Ca^{2+} elevation). It has been argued that phosphoinositide turnover would not, therefore, function as a signal (Cockroft *et al.*, 1980). However, it must be appreciated, in fact, that nonmaximal levels of a precursor (e.g., a metabolite of phosphoinositide turnover) could in principle lead to optimal product levels (e.g., Ca^{2+} elevation and/or a response such as degranulation). This would only require that the precursor pathway caused the product pathway to be saturated before the precursor pathway itself was saturated. Thus the absolute velocity of a given reaction cannot be taken as proof that it is a product or a precursor.

Evidence concerning the coupling of G proteins to the turnover of phosphatidylinositol has been obtained by Smith *et al.* (1985) and by Cockroft and Gomperts (1985). Both groups of investigators used preparations of neutrophil plasma membranes. Cockroft and Gomperts showed that membranes from cells that had been prelabeled with [^{32}P]orthophosphate and stimulated by 100 μM GTPγS hydrolyze 20–30% of their labeled PIP and PIP_2 in the presence of 100 nM Ca^{2+}. The radiolabel was quantitatively recovered

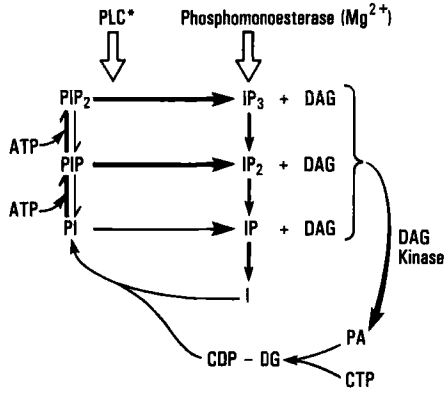

FIG. 8. Simplified scheme of phosphoinositide metabolism (adapted from Majerus *et al.*, 1984). The phosphoinositides (PI, PIP, PIP_2) are interconvertible but synthesis is presumably favored. In the presence of active PLC*, phosphoinositides are hydrolyzed, but the specificity is such that PIP_2 (and perhaps PIP) are the primary substrate when Ca^{2+} is low. The soluble headgroup inositol phosphates are sequentially degraded by a Mg^{2+}-dependent phosphomonoesterase. DAG is rapidly esterified to PA. In the presence of CTP, PA can be converted into PI. If the rate of PA production is large compared to PI production, the accumulation of PA will roughly reflect the activity in the pathway.

in the soluble inositolphosphates (IP_2 and IP_3) consistent with the action of phospholipase C (see Fig. 8). Roughly 50% hydrolysis occurred in the presence of 1 m*M* Ca^{2+} alone. These observations imply that the G protein can activate a phospholipase but do not address the role of receptor in the activation pathway.

Smith *et al.* (1985) used a pulse of [^{32}P]ATP to label the membrane phospholipids. In the presence of approximately 2 μ*M* Ca^{2+} they found that a combination of formyl peptide and 10 μ*M* GTP produced a synergistic hydrolysis of PIP_2 (30%) compared to either reagent alone (<10%). This hydrolysis was absent if the cells from which the membranes are prepared have been treated with pertussis toxin. They were unable to recover the soluble inositolphosphates, presumably because of the presence of Mg^{2+} which led to their hydrolysis by phosphomonoesterases (Downes *et al.*, 1982). Smith *et al.* (1986) have now shown that the receptor-stimulated activity is PLC by identifying the inositolphosphate products. The combination of stimulus and guanyl nucleotide reduced the Ca^{2+} requirement into the resting cell range, ~100 n*M*. Further details of the coupling between the G protein and the phospholipase have not been elucidated in the neutrophil. The lack of knowledge of these interactions arises at two levels: little direct evidence concerning the mode of the action of this G protein as well as incomplete characterization of this phospholipase C. In contrast, molecular details are

becoming available for the analogous regulation of the cGMP phosphodiesterase by G protein in the photoreceptor (Stryer, 1986).

Our present models for the details of the coupling events come, once again, from the β-adrenergic receptor (G_s/adenylate cyclase) and the photoreceptor (transducin/cGMP phosphodiesterase). The transducin interaction with cGMP phosphodiesterase is portrayed as a low amplification system in which the activated α subunit of transducin activates one or, at most, a few cGMP phosphodiesterase molecules during its lifetime. The cGMP phosphodiesterase is envisioned as being active only when coupled to activated transducin. Even so, the first two stages of activation can give rise to a three-order of magnitude amplification. Moreover, the turnover number for the phosphodiesterase is >1000/second. This enables photoreceptor response to be saturated with less than 1% activation (bleaching) of the photoreceptors. Transducin activity is presumably terminated when GTP is hydrolyzed ($t_{1/2}$ approximately 30 seconds; see Stryer, 1986). However, cGMP phosphodiesterase activity decays in about 4 seconds in the presence of ATP. It has been suggested that the protein arrestin (see Section III,E, above) undergoes an ATP–ADP exchange and that the ATP form directly inhibits the activated phosphodiesterase.

The G_s/adenylate cyclase system is less well characterized, probably in large part due to the unavailability (until recently) of purified adenylate cyclase preparations. Preparations of G_s hydrolyze GTP with a $t_{1/2}$ of seconds at 30°C (Brandt and Ross, 1985). The cyclase activation may also require continued coupling between active G_s and cyclase. The dependence of the output of cAMP on the number of receptors appears to vary among different cell types. In some systems, at least, cAMP accumulation is proportional to receptor occupancy (Mahan and Insel, 1986).

The details for the stimulation of phosphoinositide metabolism are considerably less clear. Several reports provide hints into the relationship. Pulse experiments to examine phosphoinositide turnover have been performed in the platelet. Holmsen and colleagues (1981) use a protocol in which platelets are exposed to thrombin and then free thrombin is complexed with hirudin. The addition of hirudin after thrombin was found to rapidly inhibit the net accumulation of phosphatidic acid. The results suggest that many receptors can participate in the metabolism of phosphoinositides. Preliminary results suggest that the output of the formyl peptide receptor, as measured by PA accumulation in stimulated neutrophils, is roughly proportional to the number of formyl peptide receptors occupied (Traynor and Sklar, unpublished). Taken together with the fact that most receptors may participate in activation leading to oxidant production, we imagine that the components of the activation sequence (G, PLC, phosphoinositides, etc.) are sufficient in number to accommodate amplification involving all the formyl peptide re-

ceptors. This interpretation needs to be reconciled with the observation that maximal levels of soluble inositoltrisphosphate are elicited by fewer than all of the available receptors (Dougherty *et al.*, 1984). This apparent contradiction will be discussed below where we will consider, in a semiquantitative manner, amplification at the level of PI metabolites.

Molecular details of the functioning of phospholipase C are remarkably incomplete (for example see Low *et al.*, 1984). The specificity of the enzyme or enzymes, the nature of its Ca^{2+} requirements (which may be different for individual target phospholipids) and the mode of activation and decay with respect to the G protein interaction, are just beginning to become available (Cockcroft *et al.*, 1984). The data presented by Smith *et al.* (1985) are consistent with a preference of the phospholipase for PIP_2 when Ca^{2+} is near physiological levels and when guanine nucleotides are present.

B. Targets of the Activation of Phospholipase C: Products of the Metabolism of Phosphoinositides

1. Labeling Considerations

There are several types of experimental protocols for labeling phospholipids to study their metabolism during the course of neutrophil activation. While it is inappropriate here to dwell on the relative merits of the different procedures it is worthwhile to point out that (1) direct comparison between the different labeling procedures and cell preparations is difficult; and (2) because phosphoinositide metabolism represents cyclic processes, the change in label distribution does not, in general, corresponds with the redistribution of lipid mass.

2. Alterations in the Distribution of Label in Phospholipid Species during Neutrophil Activation

The targets of PLC action (see Majerus *et al.*, 1984) in stimulated neutrophils appear to be phospholipids derived from phosphatidic acid esterified to inositol mono-, bis-, and tris-phosphates (Cockroft *et al.*, 1984). These lipids are largely comprised of species which contained saturated acyl chains in position 1 and polyunsaturated chains, particularly arachidonate, in position 2 (Cockroft and Allan, 1984). While the phosphoinositides represent a minor component of total cellular phospholipids which are highly enriched in the plasma membrane, the distribution of newly incorporated headgroup label into these species indicates that their metabolism is unusually active.

In the cycle which consumes and resynthesizes phosphoinositides (Fig. 8), a primary target even in the whole cell appears to be PIP_2. Several investigations report rapid loss of PIP_2 within a few seconds of exposure of the cells to stimulus amounting to up to 30% of the labeled PIP_2 within 30 seconds

(Volpi *et al.*, 1983, 1984; Dougherty *et al.*, 1984). The apparent maximal consumption of PIP_2 is detected prior to occupancy of all the formyl peptide receptors (Dougherty *et al.*, 1984). Following the initial reduction PIP_2 levels recover to resting levels or higher. The absolute quantity of PIP_2 consumed and regenerated during the processes is not completely characterized since the specific activity of the radiolabel probably changes during the course of the response. However, it appears that regeneration may keep PIP_2 within 75% or more of the level in resting cells during the activation sequence. Based on the observation that nearly all formyl peptide receptors can couple to the guanine nucleotide binding protein (Fig. 4) and that nearly all receptors contribute to the ultimate production of oxidant function (Fig. 2), it may be inferred that PIP_2 (as a potential substrate during signal amplification) is not likely to be limiting in the activation cascade. The regulation of PIP_2 levels, an interplay between enzymes in the plasma membrane, endoplasmic reticulum, and cytosol, is beyond the scope of this article.

The behavior of PI and PIP is somewhat more variable in the available reports. Generally, net label is lost more slowly, if at all, from PIP and PI, then label recovers in the cells to levels above the resting levels. As indicated, such differences could arise due to differences in labeling methodology or experimental conditions. We believe that even major differences in the absolute direction of labeling changes (increase vs decrease) may reflect subtle changes from system to system rather than essential differences in the biochemistry which governs label distribution. There is general accord on the observation that label in PA increases with time. Since PA may arise rapidly via synthesis from DAG and be depleted slowly by conversion into PI, it is reasonable to expect that the extent of label in PA may be roughly proportional to the throughput of the cycle (Fig. 8).

3. *Amplification via Hydrolysis of PIP_2*

A semiquantitative estimate of the quantity of the metabolites which are produced at this stage of transduction may be obtained. Neutrophils contain 50 pg protein/cell of which roughly 10% or 5 pg is associated with the plasma membrane. Plasma membrane lipid is of a similar mass and typically 0.1–0.5% is PIP_2 (10–50 attomoles/cell) and 3% is PI (300 attomoles/cell) (see Majerus *et al.*, 1984). Mass measurements in the neutrophil provide a value for PI of 500 attomoles/cell and a maximal loss of up to 100 attomoles/cell (potentially cycled into PIP and PIP_2) during stimulation in the presence of cytochalasin B (Cockroft and Allan, 1984). Because of turnover, this value must reflect a minimal estimate of the number of moles actually consumed.

If we assume that the loss of headgroup radiolabel from PIP_2 is roughly representative of the mass change in PIP_2, then maximal stimulation evokes a loss in PIP_2 of no more than 30–40% over a period of less than a minute

into a cytoplasmic volume of roughly 0.2 pl. This would represent the formation of 4–20 attomoles of IP_3 and DAG/cell. In a cytochalasin B-treated cell, a measured accumulation of DAG of 30 attomoles has been reported (Cockroft and Allan, 1984). If IP_3 were to accumulate without hydrolysis, its cytoplasmic concentration could reach 20–100 μM.

If all formyl peptide receptors (e.g., 5×10^4) were to contribute equally to PIP_2 hydrolysis, then 300 or more IP_3 and DAG per receptor would be produced per receptor. Based on the ultimate consumption of PI, the amplification could be a thousand or more (i.e., an activated PLC could cleave 1000 or more lipid headgroups). Given the likelihood that IP_3 reaches maximal levels prior to the occupancy of all the receptors, substantially greater amplification is probable. Much of this amplification is probably due to the turnover number for the phospholipase C and the lifetime of the phospholipase once it has been activated since there may be relatively little amplification in receptor coupling to G protein or G protein coupling to PLC.

4. Termination of Phosphoinositide Turnover

The phosphoinositide cycle is subject to control at least at two distinct stages: (1) the lifetime of PLC* as controlled by the transduction process (Sections III,E and IV,A) and (2) the availability of substrates for PLC. These processes and their relationship to the transduction sequence are not completely understood. It is worth reiterating the transient nature of the O_2^- response in the neutrophil which is putatively tied to this pathway. This has a half-time for decay of 15 seconds (Fig. 2). The intermediates (i.e., phosphoinositide metabolites, etc.) putatively associated with this function are likely to decay at least as rapidly as the functions themselves. We anticipate therefore that the PLC does not remain active long after the receptor which produced it decays and that the activity of any PLC molecule is normally confined to a relatively brief interval.

C. Linkage between Inositolphosphate Generation and Release of Intracellular Ca^{2+}

1. Distribution of Inositolphosphates

In the intact neutrophil, elevation of IP, IP_2, and IP_3 are all a consequence of cell activation by formyl peptides and leukotrienes (Dougherty *et al.*, 1984; Bradford and Rubin, 1985). Kinetic analysis for the formyl peptides indicates that under conditions of optimal stimulation IP_3 achieves maximal levels within the first 30 seconds, IP_2 within the first 1–5 minutes, and IP only within 5–30 minutes.

A detailed explanation for these kinetics is not available but several factors

may contribute. If the PLC were relatively specific for PIP_2, then the sequential appearance of IP_3, IP_2, and IP could result in part from the production of IP_3 and its degradation and sequential accumulation in IP_2 and IP due to phosphomonoesterase activity. If the specificity of the PLC were also influenced by the rising Ca^{2+} levels during activation (Fig. 8), then the sequential distribution could arise in part by this change in specificity. Studies in broken cell preparation indicate PLC appears to be relatively specific for PIP_2 (and PIP) in the presence of nucleotide and physiologic Ca^{2+} (Cockroft *et al.*, 1984; Smith *et al.*, 1985; Cockroft and Gomperts, 1985). It has been suggested that Ca^{2+}-dependent phospholipase D produces PA directly from PI in intact neutrophils (Cockroft, 1984; Cockroft and Allan, 1984).

2. *Roles of Inositolphosphates*

IP_3 has been implicated in the release of Ca^{2+} from intracellular stores in hepatocytes (Joseph *et al.*, 1984), pancreatic cells, (Streb *et al.*, 1983) and neutrophils (Burgess *et al.*, 1984; Prentki *et al.*, 1984) among others. Receptor-mediated increases in IP_3 occur in blowfly salivary gland, platelet, parotid, as well as the cell types above (see Marx, 1984; Berridge, 1984; Majerus *et al.*, 1984). These increases are rapid in response to agonists for diverse receptors including α-adrenergic agonists, vasopressin, 5-hydroxytryptamine, and thrombin.

As far as can be ascertained, Ca^{2+} release depends specifically upon IP_3 compared to IP_2 and IP. The efficacy of IP_3 has been defined in permeabilized cell preparations by its ability to induce the release of subcellular Ca^{2+} into the medium. Half optimal IP_3 concentrations have been reported to be in the range of 0.1 to 1 μM for hepatocytes, pancreatic acinar cells, as well as neutrophils. In hepatocyte membranes, resequestration of the released Ca^{2+} followed the degradation of IP_3. The stores of Ca^{2+} in the platelet and hepatocyte appear to be nonmitochondrial vesicular bodies perhaps related to endoplasmic reticulum. Accumulation of Ca^{2+} in these stores requires ATP (Joseph *et al.*, 1984; O'Rourke *et al.*, 1985). Specific intracellular receptors for IP_3 associated with the stores have been reported (Spät *et al.*, 1986).

3. *Amplification and Termination of the IP_3 Mediated Ca^{2+} Elevation*

Neutrophils contain in their releasable stores 40–50 pmol of $Ca^{2+}/10^6$ cells or 2.5×10^7 Ca^{2+} molecules/cell (Lew *et al.*, 1985a; Sklar and Oades, 1985). It appears that much of the Ca^{2+} elevation in the first minute following cell stimulation arises from intracellular stores. This conclusion is based upon the observation that if cells are exposed to extracellular Ca^{2+} chelators immediately prior to cell stimulation that the first 30–60 seconds of the Ca^{2+} signal are similar (Korchak *et al.*, 1984b; Sklar and Oades, 1985).

The intracellular levels of Ca^{2+} are reported to be approximately 10^{-7} *M* in the resting cell and approximately 10^{-6} *M* in the stimulated cell (Pozzan *et al.*, 1983). However, it should be appreciated that in a cell labeled with 1 m*M* cytoplasmic Quin 2, that a 20% increase in the Quin 2 signal (i.e., from 60 to 80% saturation) reflects an amount of Ca^{2+} sufficient to complex 200 μ*M* Quin 2 (i.e., 40 attomoles in 0.2 pl). If no Ca^{2+} chelators were available this would contribute a net Ca^{2+} elevation in the cytoplasm of 200 μ*M*.

Based on pulse experiments we estimated that optimal (although transient) Ca^{2+} elevation depends upon roughly 1% of the formyl peptide receptors (Sklar *et al.*, 1985a). Similar estimates would arise if the kinetics and dose–response of ligand binding were compared to the kinetics and dose–response of Ca^{2+} elevation. If 1% of the receptors (500) contribute to the initial release of 2.5×10^7 Ca^{2+}, then each receptor contributes an average of 50,000 Ca^{2+}. These Ca^{2+} are presumably released relatively near the site of receptor activation since cell activation appears to be localized to regions near the sites of ligand binding (Ohno *et al.*, 1982a,b; Sawyer *et al.*, 1985). The absolute distances for diffusion of the Ca^{2+} signal depends not only on the Ca^{2+} diffusion but on the rate that G* diffuses from the receptor, PLC, and lipid diffusion in the membrane, and IP_3 diffusion in the cytosol compared to the rates at which all of these Ca^{2+} precursors are terminated.

The absolute quantities of IP_3 generated appear to be well within the range necessary for stimulation of Ca^{2+} release. The total output of IP_3 is predicted to approach 100 μ*M* if there were no hydrolytic phosphomonesterase activity. If all receptors were equally coupled to PIP_2 hydrolysis, 1% of the receptors would provide 1 μ*M* IP_3. On a receptor basis therefore we are led to the tentative conclusion that each receptor produces >300 IP_3 in the vicinity of the activated receptor which is adequate to release approximately 50,000 Ca^{2+}. These factors of amplification are not without precedent since a single photon in the photoreceptor can lead to the hydrolysis of up to 10^6 cGMP per cell per second (Stryer, 1986). These observations point to the fact that some signals are generated without occupying all of the receptors.

This apparent excess in the generating capacity for signals is an element of some interest. The Ca^{2+} signal is only maximal for seconds if cells are stimulated with a 1% pulse of receptor occupancy (Sklar *et al.*, 1985a). Thus while the first percent of occupancy leads to the release of the stores, subsequent IP_3 which is generated presumably maintains Ca^{2+} outside of the stores. That is, as Ca^{2+} is resequestered, the excess IP_3 may be used for its rerelease. Following the initial Ca^{2+} release the additional IP_3 would appear to provide quantitatively inefficient (i.e., nonlinear) release due to a lack of Ca^{2+} available for rerelease.

Regulation of Ca^{2+} stores is envisioned to represent an interplay among

IP_3 generation, IP_3 degradation, and Ca^{2+} resequestration and other pump activities. It is not clear whether Ca^{2+} levels fail at the end of activation because (1) IP_3 levels fall in response to the termination of its generation or the completion of its degradation, (2) the targets of IP_3 in the stores become insensitive to IP_3, (3) the Ca^{2+} uptake mechanisms are stimulated as part of the activation process, or (4) Ca^{2+} is largely pumped out of the cell. Pulse binding experiments can provide conceptual insights into some of these possibilities. We have varied the number of receptors occupied during pulse stimulation and have found that the decay of the Ca^{2+} transient is relatively independent of the number of receptors which contributed to the signal (Sklar *et al.*, 1985a). If decay had been a stimulated event we would have anticipated that the decay kinetics would have been sensitive to the number of receptors occupied. The decay kinetics exhibit a constant half-time of 15 seconds at 37°C. It remains to be determined whether IP_3 is consumed prior to the decay of the Ca^{2+} signal. The pump activities are also now beginning to be characterized (Ochs and Reed, 1983; Lagast *et al.*, 1984a,b).

D. Activation and Translocation of Protein Kinase C

The expression of the appropriate kinase activities in cells is believed to be a critical feature of the activation sequence. The intimate details of these processes as they relate to protein kinase C are beginning to be understood (Nishizuka, 1984). The regulation of protein kinase C in stimulated neutrophils appears to require an interplay among its redistribution in the cytosol, activation at its redistributed loci, modulation of its activation, and the accessibility of its own molecular binding sites to the appropriate substrates (e.g., ATP) and ligands or cofactors (e.g., DAG, arachidonic acid, Ca^{2+}, PS). A summary of some of the proposed regulatory features is included in Fig. 5.

1. Kinase Activities in Neutrophils

Among the kinases found in neutrophils are cAMP-dependent, cGMP-dependent, and Ca^{2+} phospholipid-dependent activities, the last of which appears to be the most prominent (Huang *et al.*, 1983; Helfman *et al.*, 1983a,b). Protein kinase C may be characterized either according to its binding for phorbol esters or its ability to phosphorylate proteins either *in situ* or in a synthetic assay where the substrate is a histone. The PKC in resting neutrophils is reported to be either largely cytosolic (Wolfson *et al.*, 1985) or distributed between cytosolic and particulate fractions (Helfman *et al.*, 1983a,b). Variation in the results may arise from procedural differences in the preparation of the fractions (especially the presence of Ca^{2+} or Ca^{2+} chelators), differences in the assay procedures (which may not be comparable among different preparations), and potentially the presence of endogenous PKC inhibitors (Balazovich *et al.*, 1985). It appears that a characteriza-

tion of the PKC activity of the neutrophil and its distribution is still at a preliminary stage. PKC is known, however, to have a requirement for Ca^{2+} and PS for its association with lipid membranes. Neutrophil PKC is activated in the presence of Ca^{2+} and phospholipid by DAG or by arachidonic acid (McPhail *et al.*, 1984a) and is also a target of stimulation by retinoids (Badwey *et al.*, 1985).

Redistribution of PKC activity is seen in neutrophils in the presence of phorbol esters (Wolfson *et al.*, 1985), Ca^{2+} ionophore, and formyl peptide (Dougherty and Niedel, 1985). A loss of cytosolic kinase activity following the treatment of cells with PMA (ED_{50} approximately 40 n*M*) is accompanied by the appearance of PKC activity in the particulate fraction of a cell sonicate and the recovery of enhanced NADPH oxidase activity (Wolfson *et al.*, 1985). The redistribution of the kinase precedes in time the activation of the NADPH oxidase and has been suggested to be responsible for it.

Less detailed accounts of the kinase redistribution induced by Ca^{2+} ionophore or by formyl peptide are presently in the literature. Ca^{2+} ionophore leads to redistribution of the binding activity for PdBu as well as kinase activity. Preliminary reports (Dougherty and Niedel, 1985) indicate redistribution in the presence of formyl peptides, but the kinetics of redistribution and its dose–response with respect to receptor occupancy have yet to be completely elucidated.

2. *Roles of Protein Kinase C Activation in Neutrophils*

Phorbol esters, which appear to be highly specific ligands for protein kinase C, lead to activation of the NADPH oxidase in neutrophils, the long-lived production of oxidants, extensive degranulation, and dramatic changes in cell morphology. Activation by PMA occurs even when cytosolic Ca^{2+} is depleted to levels below 10 n*M* (DiVirgillio *et al.*, 1984) but is sensitive to calmodulin inhibitors (Robinson *et al.*, 1985). There is synergy between weakly stimulatory Ca^{2+} increases induced by ionophore and weakly stimulatory PMA concentrations. Together, they amplify the rate of oxidant production and decrease its latency period (Robinson *et al.*, 1984). Other combinations of stimuli also act synergistically including the combination of DAG which stimulates kinase and formyl peptide which both lead to stimulated Ca^{2+} elevation and translocates kinase (Dewald *et al.*, 1984; Dewald and Baggiolini, 1985). Observations of synergy have also been made in subcellular fractions from stimulated cells. Oxidase activation by PKC activators was "primed" by sequential application of different activators (McPhail *et al.*, 1984b).

Together these observations have led to the notion that translocation and activation of C kinase were ultimately responsible for neutrophil activation both by PMA and membrane receptors including the formyl peptide receptors. This general principle has, however, been recently called into question

by the work of Gerard *et al.* (1986). These investigators synthesized a novel isoquinoline sulfonamide [1-(5-isoquinolinesulfonyl)piperazine] a member of a family of drugs which has been shown to exert inhibitory effects on mammalian protein kinases by competitively binding to the ATP substrate site (Hidaka *et al.*, 1984). This new inhibitor has a K_I of 20 μM for PKC but also a K_I of 3 μM for cAMP-dependent kinase. It was found to inhibit the normal oxidative response to PMA and to a synthetic diacylglycerol analog, 1-oleyl-2-acetyl glycerol. It does not inhibit the response to C5a or formyl peptide. These observations have been interpreted to indicate that formyl peptide-induced activation of PKC is not the primary pathway of neutrophil activation. Other investigators have observed that PKC inhibitors fail to inhibit neutrophil activation by receptor–ligand interaction, Ca^{2+} ionophore and PMA (Wright and Hoffman, 1986) while calmodulin antagonists are inhibitory to cell function (Wright and Hoffman, 1986; Robinson *et al.*, 1985; Jones *et al.*, 1982). These results could indicate the importance of calmodulin-dependent processes in cell activation but may also indicate that *in vitro* assays of PKC and its inhibitors are not analogous to the intracellular stimulation of PKC activity.

3. *Targets of Protein Kinases*

A number of neutrophil proteins become phosphorylated during the course of cell activation (Schneider *et al.*, 1981; Huang *et al.*, 1984; White *et al.*, 1984; Papini *et al.*, 1985; Segal *et al.*, 1985) including vimentin (Huang *et al.*, 1984) and myosin light chain (Fechheimer and Zigmond, 1983). In general, the relationships of phosphorylation to receptor occupancy, the pathways responsible for the phosphorylation, and the role of the proteins in cell activation have yet to be entirely elucidated.

The phosphorylation of a 44 kDa species is absent in cells from chronic granulomatous disease patients that fail to generate an oxidative burst (Segal *et al.*, 1985). The phosphorylation of this species in normal cells follows the time course of the oxidative burst and is suggested to be associated with it. Whether this species is responsible for activation, termination, or some other role is not known.

4. *A Model for the Role of Ca^{2+} and DAG in the Activation and Translocation of PKC*

The binding of PKC to membrane is Ca^{2+} dependent and sensitive to Mg^{2+}. In a model system using erythrocyte membrane vesicles and purified PKC (Wolf *et al.*, 1985) it was found that PKC association with membrane occurs rapidly ($t_{1/2}$ approximately 30 seconds) in the presence of μM Ca^{2+} concentration. When Ca^{2+} is lowered into the nM range with EGTA, PKC dissociates rapidly ($t_{1/2} < 30$ seconds). The presence of Ca^{2+} also increases the sensitivity of PKC to translocation from soluble to membrane bound

form by PdBu (Wolf *et al.*, 1985). These synergistic interactions are included in Fig. 5.

5. *Amplification of a PKC Signal*

Amplification of PKC activity appears to require both a Ca^{2+}-dependent translocation and an activation step by a lipid mediator. The Ca^{2+} release depends initially upon 1% of the receptors while sustained release requires additional receptors (e.g., approximately 10% over 30 seconds). Since Ca^{2+} diffusion in the cytoplasm is likely to be restricted (Sawyer *et al.*, 1985) then the accessibility of soluble proteins (e.g., PKC) to newly released Ca^{2+} may depend in part upon their own diffusion in the cytoplasm. The cytosolic distribution of PKC with respect to the plasma membrane is not well defined, however, we anticipate that a maximal PKC translocation will require not only maximal Ca^{2+} elevation but Ca^{2+} elevation which is sustained for a moderate period of time. In this connection, it is useful to recall that a maximal rate of oxidant production elicited by formyl peptide receptors requires $\geq$10% of the receptors and a time $\geq$30 seconds.

Based on the binding of radioactive PdBu, there are approximately 4×10^5 PKC/neutrophil (Goodwin and Weinberg, 1982) which would be hypothetically activated by approximately 10% of the receptors. If each receptor leads to the hydrolysis of 10^2–10^3 PIP_2, then 10% (5000) of the receptors could easily lead to production of 500,000 DAG. Whether all these DAG will be available for PKC activation is not known. Presumably, the accumulation of DAG will depend upon its rates of production and destruction either by turnover (by DAG kinase) or hydrolysis (DAG lipase). The activity of DAG kinase is not well characterized but competition for DAG with PKC cannot be ruled out. One report also suggests a cytoskeletal association for DAG kinase in chick embryonic muscle (Daleo *et al.*, 1966) which could in principle render DAG levels sensitive to cytoskeletal modulators. Indeed, cytochalasin B has been reported to augment DAG levels in stimulated neutrophils (Honeycutt and Niedel, 1986). Nonetheless, it appears that the output of a small fraction of the receptors should be sufficient to maximally activate PKC. Since Ca^{2+} and DAG levels would decline after maximal elevation following occupancy of only a few percent of the receptors, we anticipate that most formyl peptide receptors contribute to sustaining a maximal activation of PKC.

The rise in cytosolic Ca^{2+} may also have a distinct set of effects on PKC regulation. The specificity of the PLC may be influenced by Ca^{2+} so that additional substrates (PI as well as PIP and PIP_2) may become available for DAG production. The potential activation of other lipases such as phospholipase A2 and diglyceride lipase by Ca^{2+} is expected to generate arachidonate from phospholipid and DAG, respectively.

6. *Termination of the Kinase Activation*

Within a few minutes following stimulus, the rate of ligand binding decreases since cytoplasmic receptors are inserted relatively slowly into the membrane (Zigmond *et al.*, 1982). As the binding rate falls, occupied receptors should become inactive, the rates of signal generation should decline, and the signal levels should decay. As Ca^{2+} falls from μM to 100–200 nM and as DAG is either recycled to PA or hydrolyzed, PKC is expected to be released rapidly from the membrane and inactivated. In pulse stimulation, Ca^{2+} and O_2^- begin to decay in parallel following a delay of 5 seconds (Omann *et al.*, 1985). Since oxidant production (a putative result of PKC activation) and Ca^{2+} (a putative precursor of PKC activation) decay in parallel it would appear that PKC release need not be the rate-limiting step in the decay of the oxidase. There could be an independent mechanism regulating oxidase activity so that the oxidase system would be intrinsically short-lived in the absence of sustained cell activation.

V. Implications

A. Amplification

In the pathway of events which can be linked to receptor occupancy we tentatively suggest the following sequence of amplification (Fig. 9). Since oxidant production is nearly proportional to formyl peptide receptor occupancy (Fig. 2) and since most formyl peptide receptors appear to have access to coupling proteins (Fig. 4), we hypothesize that coupling protein is not limiting in cell activation. The stoichiometry of the components is such that massive amplification at this first stage seems unlikely.

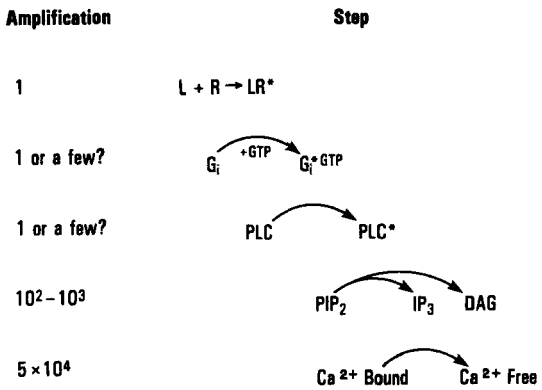

FIG. 9. Hypothetical scheme for amplification in neutrophil transduction and signal generation (inspired by Stryer *et al.*, 1981).

Based on the photoreceptor system where continued contact between activated transducin-GTP and PLC may be required, we envision this second stage of G_p activation of PLC to be a low amplification step. However, if PLC is active for the duration of G* (prior to GTP hydrolysis), a large net amplification of DAG and IP_3 will still be accomplished. The degree of amplification will depend upon the lifetime of the PLC and its turnover number.

The output of DAG and IP_3 appears to be approximately 10^2–10^3 per receptor, certainly within reason for even a single PLC activated over a period of seconds. Ca^{2+} amplification is in the range of 5×10^4 per receptor until the sequestered Ca^{2+} is entirely released by approximately 1% of the receptors. PKC activation is envisioned to require an estimated 10% of the receptors (5000) which will (1) release Ca^{2+} and sustain the elevation while PKC binds to the plasma membrane and (2) provide sufficient DAG to activate 4×10^5 PKC.

Pathways to other cell responses are less well defined. In cytochalasin B-treated cells, approximately 10% of the receptors lead to optimal degranulation. In nontreated cells less than 100 receptors lead to an optimal (albeit transient) polymerization of actin. The amplification of this response, through an as yet unknown mechanism is phenomenal, approximately 10^5 G-actins incorporated into F-actin per receptor (Sklar *et al.*, 1985b; Omann *et al.*, 1986a).

Each receptor could, in principle, also lead to the production of $\geq 10^5$ O_2 (i.e., >10 nmol $O_2^-/10^6$ cells at receptor saturation). In order to understand the amplification leading to this response it will be necessary to know the turnover number for the kinase (i.e., the number of oxidases activated for each kinase) and the turnover number for each oxidase (the rate of O_2^- production by an oxidase times the duration of activation of an oxidase). These details should be forthcoming. We presume that such massive amplification is possible only because of the cascade of events intervening receptor occupancy and the generation of cell function. We suggest that a direct interaction between transiently activated receptors and functional effectors (i.e., oxidase molecules) would not seem a priori to be able to account for such amplification.

B. Transient Responses and Termination Steps

At least 6 cell responses (some of them associated with one another) decay or stop rapidly following the inhibition of ligand binding in pulse stimulation (Sklar *et al.*, 1985a). In neutrophils these include Ca^{2+} elevation, O_2^- production, and actin polymerization which appears to be associated with light scatter, membrane ruffling, and transient aggregation under nondegranulating conditions. In addition, in platelets stimulated by a pulse of thrombin,

granule enzyme secretion can be inhibited following a pulse as well as the accumulation of PA. If we assume that PA reflects throughput in the phosphoinositide cycle, this would imply that sustained PIP_2 hydrolysis and IP_3 and DAG production also decay rapidly. Based on the scheme for PKC activation, we would predict that PKC may rapidly dissociate from the membrane and return inactive to the cytosol following pulse stimulation.

In a tentative pathway which leads from membrane receptor through cell signals and gives rise to oxidase activation via PKC-dependent phosphorylation, there appears to be a minimum of eight components of activation: (1) receptor activation, (2) G-protein activation, (3) PLC activation, (4, 5) hydrolysis of PIP_2 and IP_3 and DAG, (6) IP_3-mediated Ca^{2+} elevation, (7) synergistic PKC activation and translocation by Ca^{2+} and DAG (and/or activation of calmodulin dependent kinases), and (8) phosphorylation of enzyme(s) in the oxidase system. We envision that each of these steps requires a specific mechanism of termination.

1. LR* decays into LR_0, an interaction regulated in part by G proteins, but potentially also by receptor phosphorylation or cytoskeletal attachment which could influence the accessibility of LR to G.
2. G* should activate PLC prior to GTP hydrolysis (for example, $t_{1/2}$ is 30 seconds for transducin and a few seconds for G_s).
3. PLC could remain active while in contact with G*, but like the cGMP phosphodiesterase in the photoreceptor, there could be an additional specific termination step.
4. DAG is lost via hydrolysis (DAG lipase) or recycled into PA by DAG kinase. This latter pathway may be highly efficient as indicated by the accumulation of labeled PA during cell activation.
5. IP_3 is rapidly hydrolyzed (seconds?), presumably by Mg^{2+}-dependent phosphomonoesterases.
6. Ca^{2+} resequestration occurs in seconds.
7. PKC inactivation would occur in the time frame of DAG and Ca^{2+} loss but may also be subject to specific PKC inhibitors.
8. Oxidase activation may be rapidly reversed (by dephosphorylation?).

The rate-limiting steps in the decay process are uncertain. Since both Ca^{2+} and O_2^- decay in parallel following pulse stimulation, we suspect that specific termination steps make possible a more rapid loss of oxidant production than would occur if the entire activation sequence had to decay in a stepwise fashion.

There is a remarkable inhibition of oxidant production which occurs when neutrophils which had been stimulated with formyl peptide are exposed to activators of receptors which are coupled to G_s and adenylate cyclase (β-adrenergic agonists and PGE_1) (Lad *et al.*, 1985a). The oxidant output of

50,000 form peptide receptors can be shut down by <1000 β-adrenergic receptors (Tecoma *et al.*, 1986). Step 2 need not be a target for inhibitory receptors since transduction per se is not obliterated by β-adrenergic agonists (Omann *et al.*, 1985; Tecoma *et al.*, 1986). There is nearly normal actin polymerization and cell aggregation under conditions where oxidant production is completely shut down. Step 3, phosphoinositide metabolism, is somewhat sensitive to β-adrenergic agonist. This suggests that G_s, working perhaps through cAMP-dependent kinases, would have an inhibitory impact on an initiation step (e.g., PLC activation) in the neutrophil activation sequence.

C. Branchpoints in a Transient Activation Sequence

The existence of branchpoints in an activation sequence has a number of noteable implications. Functions elicited by the same or different stimuli may be independently regulated (e.g., oxidant production and aggregation, Whitin and Cohen, 1985), or may be specifically inhibited (i.e., drug effects on specific functions). We suggest that certain features of cell activation can be interpreted in terms of branchpoints.

1. Signal Independent Responses

The overall regulation of Ca^{2+} occurs at steps 5 and 6 distal to receptor, G* and PLC (steps 1–4). Since PLC activation should not require an elevation of intracellular Ca^{2+}, functions which depend upon DAG or IP_3 or G* or PLC could be independent of Ca^{2+}. Indeed, cells depleted of Ca^{2+} activate cytoskeleton normally and chemotax in response to surface receptor activation while oxidant production and degranulation are restricted (Sklar *et al.*, 1985b; Sklar and Oades, 1985; Meshulam *et al.*, 1985). Under some conditions phagocytosis also appears to be Ca^{2+} independent (Lew *et al.*, 1985b).

2. Inhibitory Signals

The β-adrenergic receptors appear to target the activation sequence in a manner which recognizes a branchpoint distinguishing oxidant production and cytoskeletal activation. This branchpoint would theoretically be at or prior to the regulation of Ca^{2+} implying that some aspects of PI metabolism are less sensitive to adrenergic receptors or that there is an activation branchpoint at an even higher level. Prostaglandin and adenosine receptors also appear to generate inhibitory signals (Fantone *et al.*, 1983, 1984; Cronstein *et al.*, 1985; Lad *et al.*, 1985a).

3. Synergism

If a step below a branchpoint (e.g., PKC) is a target for several steps above (e.g., DAG and Ca^{2+}) synergism may result. A reagent which elevates the

level of either DAG or Ca^{2+} may act synergistically with a reagent which elevates the other.

4. *Simultaneity of Transient Signals*

In a cell depleted of Ca^{2+}, ligand binding is expected to transiently activate steps 1–5 without leading to oxidant production or degranulation. If Ca^{2+} is provided during the lifetime of the activation pathway, cell function can be initiated (Sklar and Oades, 1985; Valone and Johnson, 1985). This observation implies that if several transient signals following a branchpoint are all required, they must be present simultaneously to elicit optimal cell function. The requisite timing of signals would depend upon the relative decay rates for the signals.

5. *Rate of Ligand Binding*

The magnitude of various signals has been discussed in terms of their rate of generation and their rate of decay. The rate of generation in turn depends upon the rate of ligand binding and the amplification at each stage. If there are branchpoints leading to individual signals which decay at different rates, or if there are synergistic steps then the accumulation of signals along different pathways or at the synergistic steps can be sensitive to the rate of ligand binding. Indeed, it appears that neutrophils may be expected in principle to chemotax optimally at rates of receptor occupancy which do not lead to optimal oxidant production (Sklar *et al.*, 1981b; DeTogni *et al.*, 1985a; Omann and Sklar, 1985). Intracellular signals including the metabolism of PI (DeTogni *et al.*, 1985b) and the elevation of Ca^{2+} (Omann and Sklar, 1985) are sensitive to the rate of stimulus binding.

D. A Curious Result

Based on their sensitivity to pertussis toxin, it is presumed that several neutrophil chemoattractant activators make use of parallel if not identical activation pathways via coupling proteins. However, it is widely reported that leukotrienes are relatively inefficient activators of neutrophils compared to formyl peptides. The magnitude and duration of several cell functions (Palmblad *et al.*, 1984; Prescott *et al.*, 1984; Dewald and Baggiolini, 1984; Omann *et al.*, 1986b) and several cell signals (Volpi *et al.*, 1984; Naccache *et al.*, 1984; Andersson *et al.*, 1985; Goldman *et al.*, 1985b; Omann *et al.*, 1985b) appear to be diminished for LTB_4. While it is likely that the magnitude of the functions is dependent upon the magnitude and duration of the cell activation signals, there appears to be no clear explanation for the differences in efficacy between formyl peptides and leukotrienes. In principle the difference could arise because of intrinsic individuality of the ligands in their ligand–receptor interactions or in the receptor-coupling protein interactions (Omann *et al.*, 1986b). Explaining this fascinating result promises

to provide significant insight into the subtle details of the cell activation mechanisms.

E. Conclusion

There is a remarkable ferment in the molecular understanding of signal transduction in many cell types. In the neutrophil, information is becoming available which is beginning to permit an analysis of the most intimate details of amplification and termination of the biochemical pathways which lead to cell activation. While many of the biochemical details remain to be elucidated, analogies can be made with other receptors and signaling systems.

The neutrophil is a remarkable cell type, exhibiting diverse and fascinating functions, distinct receptors for many classes of stimulatory molecules, and sensitivity to the conditions of stimulus application. In this article we have attempted to provide a conceptual and quantitative outlook upon future developments which will encompass biochemical, biophysical, and physiological investigations.

Many biochemical pathways have yet to be specifically integrated into the pathways considered here. These include (1) multiple products of phosphoinositide metabolism, (2) intracellular actions of autocoids such as PAF, LTB_4, and other products of arachidonate metabolism, (3) fluxes of ions other than Ca^{2+}, (4) methylation, (5) roles of the kinases other than C kinase and the conditions which require them, (6) the roles of calmodulin, and (7) other pathways not yet studied.

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ADVANCES IN IMMUNOLOGY, VOL. 39

Arachidonic Acid Metabolism by the 5-Lipoxygenase Pathway, and the Effects of Alternative Dietary Fatty Acids

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I. Introduction

Interest in the potentially beneficial effects of supplementing the western diet with marine lipids has been generated by the evidence suggesting that the natural history of hypertensive and thrombotic disorders may be altered by modifying the availability of the eicosanoid precursor, arachidonic acid, relative to that of alternative fatty acids (Bang and Dyerberg, 1972; Dyerberg *et al.*, 1975, 1978; Bang *et al.*, 1976; Dyerberg and Bang, 1979). Greenland Eskimos and Japanese people have a high dietary intake of the long chain N-3 polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DCHA) in seafood and a low instance of myocardial infarction when compared to their western counterparts (Dyerberg *et al.*, 1978). In Japan the lowest death rates from coronary heart disease are found on the island of Okinawa where fish consumption is about twice as high as that on the mainland of Japan (Kagawa *et al.*, 1982). In the Chiba prefecture in Japan, the mortality from ischemic coronary heart disease is significantly lower in a fishing village, where the average fish consumption is about 250 g/person/day, than in a farming village, where the average fish consumption is about 95 g/person/day (Hirai *et al.*, 1980). These epidemiologic data suggest that fish consumption may protect against coronary heart disease. Moreover, a recent epidemiologic study carried out in Zutphen, Netherlands, demonstrated an inverse dose–response relationship between fish consumption in 1960 and death from coronary heart disease during 20 years of follow up (Kromhout *et al.*, 1985). Mortality from myocardial infarction was more than 50% lower among those individuals who consumed at least 30 g of fish daily as compared to those who did not eat fish. The beneficial effects of fish oils on ischemic heart disease have been attributed to the effects of EPA on plasma cholesterol and triglyceride levels and on the attenuation of the cyclooxygenase pathway and certain products so as to modify platelet and endothelial cell interactions in a favorable direction.

That dietary supplementation with fish oils leads to a hypolipidemic effect

is well documented (Harris and Connor, 1980). Salmon oil rich in N-3 decreases the levels of plasma triglycerides and very low-density lipoproteins without affecting the levels of high-density lipoproteins in normal individuals (Harris *et al.*, 1983). In patients with Type IIB and Type V hyperlipidemias (Phillipson *et al.*, 1985), dietary fish oils lead to a decrease in plasma cholesterol, triglycerides, very low-density lipoproteins, and apoprotein E levels. N-3 fatty acids also competitively inhibit the generation of the prostaglandins (PG) and thromboxane A_2 by the cyclooxygenase pathway (Needleman *et al.*, 1979; Corey *et al.*, 1983). In addition, to the extent that it is formed, the thromboxane A_3 derived from EPA by the cyclooxygenase pathway has attenuated activity relative to thromboxane A_2 in causing aggregation of human platelets (Whitaker *et al.*, 1979). Inhibition of the generation of thromboxane A_2 and substitution in part by thromboxane A_3 are believed to reduce platelet aggregation and vasospasm. The bleeding time is more prolonged and the platelet aggregation response to adenosine diphosphate (ADP) is reduced in Greenland Eskimos as compared to Danish volunteers (Dyerberg and Bang, 1979).

In a prospective study, the provision of mackerel, which is rich in N-3 polyunsaturated fatty acids, as a sole source of dietary fat reduced the formation of the proaggregatory vasoconstrictive thromboxane A_2 and inhibited platelet aggregation (Seiss *et al.*, 1980). Supplementation of the diet with 40 ml/day of cod liver oil, which provides about 4 to 5 g of EPA and about 5 to 6 g of DCHA daily, increased the bleeding time and decreased the platelet count, suppressed the platelet aggregation response to ADP and collagen stimulation, and reduced the associated formation of immunoreactive thromboxane B, which is a metabolic product of thromboxane A and serves to quantitate the active product (Lorenz *et al.*, 1983). Thirty volunteers, who ingested EPA (10 g/day) for 6 weeks, demonstrated a decrease in serum thromboxane B levels to 35% of control values by 6 weeks (Fitzgerald *et al.*, 1986). This is contrasted with the rapid effect of a single aspirin tablet (100 mg), which completely inhibited the serum thromboxane B_2 levels within 1 hour (Fitzgerald *et al.*, 1983). In patients with severe atherosclerotic vascular disease, EPA supplementation (10 g/day for 1 month) reduced excretion of the major urinary metabolite of thromboxane B_2, 2,3-dinor thromboxane B_2, and only slightly increased dinor thromboxane B_3 excretion (Fitzgerald *et al.*, 1986). The suppression of the formation of thromboxane compounds by EPA is in contrast to the effects of this polyunsaturated fatty acid on prostacyclin (PGI_2) generation by vascular endothelial cells. Fischer and Weber (1984) demonstrated that 2,3-dinor 6-keto-$PGF_{1\alpha}$ (PGI_2-M), a major urinary metabolite of PGI_2 excretion, was maintained and PGI_3-M formation was increased after feeding in healthy volunteers. In addition, PGI_3 retains the biologic properties of PGI_2 in that it inhibits platelet ag-

gregation and relaxes vascular smooth muscle *in vitro* (Fischer and Weber, 1984). Thus, dietary supplementation with EPA increases the formation of the biologically active PGI_3 and maintains PGI_2 biosynthesis while diminishing thromboxane A_2 generation and producing some functionally attenuated thromboxane A_3. This combination of events would be expected to result in an inhibition of platelet deposition on vascular surfaces *in vivo* without impairing the vascular response of other stimuli.

Thus, previous clinical investigation has focused exclusively on the effects of N-3 fatty acids in the modulation of the metabolism of arachidonic acid by the cyclooxygenase pathway. Because arachidonic acid released from membrane phospholipids can also be metabolized by lipoxygenase pathways, of which the 5-lipoxygenase pathway leads to the generation of the proinflammatory leukotrienes, we will review the recent studies of the effects of EPA and DCHA on leukotriene generation and function. In order to appreciate the possible effects of alternative fatty acids on the function and products of the 5-lipoxygenase pathway, it is appropriate to consider the steps in biosynthesis and metabolism of the leukotrienes, the nature of subclass-specific receptors, and the pharmacologic and likely physiologic actions of the leukotrienes.

II. Biosynthesis and Metabolic Inactivation of Arachidonic Acid-Derived Mediators

Arachidonic acid released from membrane phospholipids by the actions of phospholipase A_2 (Bills *et al.*, 1977; Lapetina, 1982; Lapetina and Seiss, 1983; Seiss and Lapetina, 1983), or by the combined action of a phosphoinositide-specific phospholipase C_1 and a 1,2-diacylglycerol lipase (Majerus, 1983; Majerus *et al.*, 1983; Neufield and Majerus, 1983), during cell activation may be reesterified or oxidatively metabolized by the enzymes of the cyclooxygenase or lipoxygenase pathway. The cyclooxygenase pathway leads through intermediates to the formation of thromboxane A_2 and prostaglandins. The 5-lipoxygenase generates 5*S*-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HPETE) (Borgeat and Samuelsson, 1979a) that is converted by the same 5-lipoxygenase to 5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (leukotriene A_4, LTA_4) (Borgeat and Samuelsson, 1979b; Hammarström and Samuelsson, 1980; Rådmark *et al.*, 1980; Shimuzu *et al.*, 1984; Rouzer *et al.*, 1986). Alternatively, 5-HPETE is reduced to its alcohol 5-hydroxy,6,8,11,14-eicosatetraenoic acid (5-HETE). LTA_4 is processed by an epoxide hydrolase to 5*S*,12*R*-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid (leukotriene B_4, LTB_4) (Borgeat and Samuelsson, 1979c) or by a novel glutathione *S*-transferase termed LTC_4 synthetase to 5*S*-hydroxy-6*R*-*S*-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (leukotriene C_4, LTC_4) (Murphy

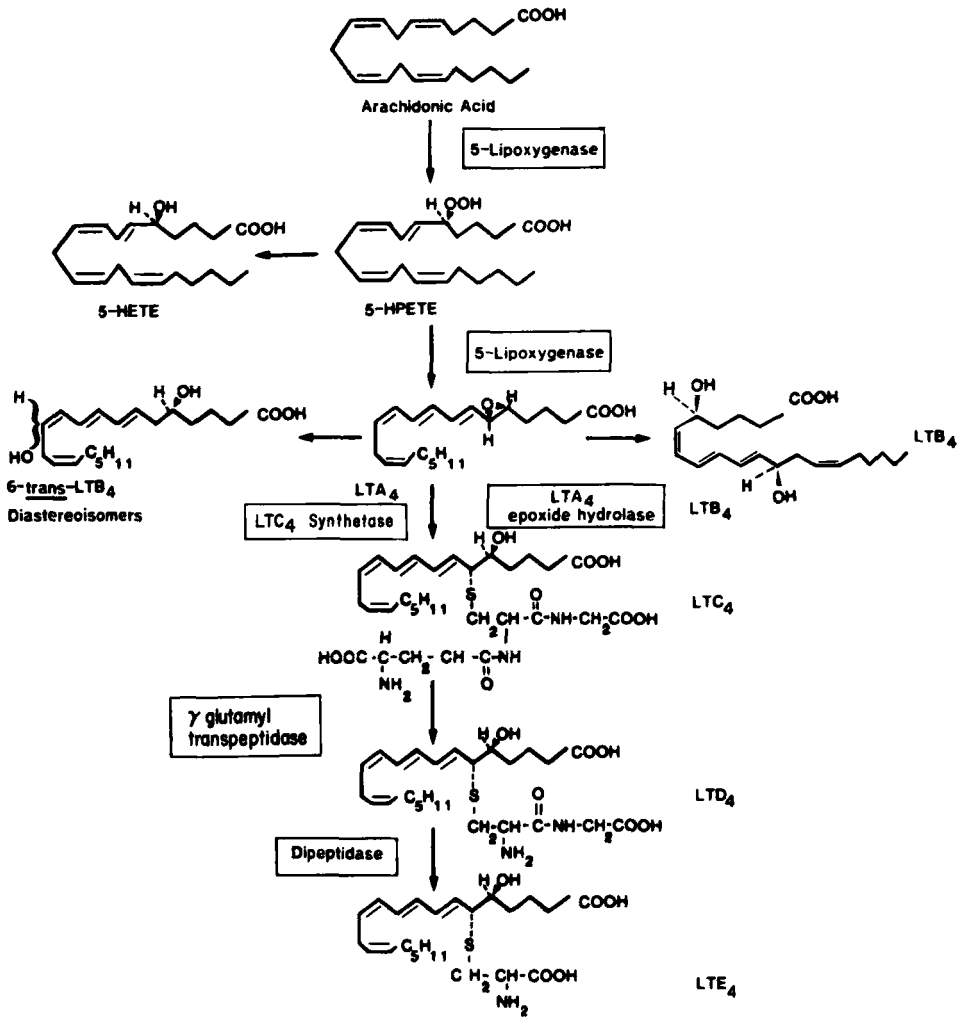

FIG. 1. Metabolism of arachidonic acid (AA) by the 5-lipoxygenase pathway. The full nomenclature for each product is in the accompanying text.

et al., 1979; Bach *et al.*, 1980, 1984; Yoshimoto *et al.*, 1985). LTC_4 is cleaved by γ-glutamyl transpeptidase to 5*S*-hydroxy-6*R*-*S*-cysteinylglycyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (leukotriene D_4, LTD_4) and by a dipeptidase to 5*S*-hydroxy-6*R*-*S*-cysteinyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (leukotriene E_4, LTE_4) (Murphy *et al.*, 1979; Bach *et al.*, 1980; Lewis *et al.*,

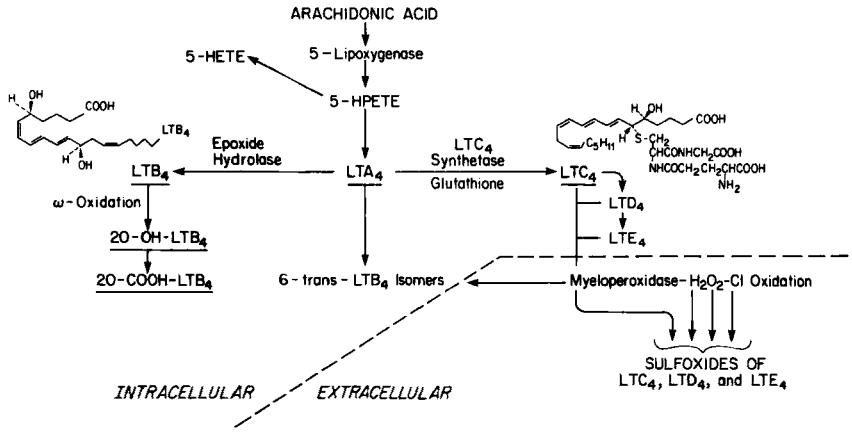

FIG. 2. Diagrammatic representation of biosynthesis and metabolism of the sulfidopeptide leukotrienes and LTB_4.

1980a,b; Morris *et al.*, 1980; Orning *et al.*, 1980). LTA_4 also undergoes nonenzymatic hydrolysis to 5*S*,12*R*- and 5*S*,12*S*-dihydroxy-6,8,10-*trans*-14-*cis*-eicosatetraenoic acid diastereoisomers (5*S*,12*R*- and 5*S*,12*S*-dihydroxy-6-*trans*-LTB_4, respectively) (Fig. 1) and to minor products, 5,6-dihydroxy-eicosatetraenoic acid diastereoisomers (Borgeat and Samuelsson, 1979d).

In addition to the bioconversion of LTC_4 to LTD_4 and to LTE_4, there is another mechanism for modifying the structures and functional activities of the sulfidopeptide leukotrienes, which depends upon the triggering of the respiratory burst (Fig. 2) (Lee *et al.*, 1983). Human neutrophils (PMN) and eosinophils metabolize LTC_4, LTD_4, and LTE_4 through an extracellular H_2O_2-peroxidase-chloride-dependent reaction to 6 products that elute as three doublets on reverse-phase high-performance liquid chromatography (RP-HPLC). More than 90% of the metabolites are composed of the 6-*trans*-LTB_4 diastereoisomers and the subclass-specific diastereoisomeric leukotriene sulfoxides. The 6-*trans*-LTB_4 diastereoisomers are inactive as spasmogenic agents and are not immunoreactive in a sulfidopeptide leukotriene radioimmunoassay. The sulfoxides possess <5% of the spasmogenic activity but are fully immunoreactive. Leukotriene B_4 is metabolized in human PMN to the substantially less active omega metabolites, 20-hydroxy- and 20-carboxy-LTB_4, respectively (Hansson *et al.*, 1981; Camp *et al.*, 1982) by a sequence that involves a unique microsomal cytochrome, *P*-450, followed by cytosolic steps (Soberman *et al.*, 1985b).

III. Specific Enzymes in Leukotriene Biosynthesis and Processing

A. The 5-Lipoxygenase

The 5-lipoxygenase enzyme is located in the cytosol of rat basophil leukemia (RBL-1) cells, guinea pig PMN, and human PMN (Jakschik and Lee, 1980; Furukawa *et al.*, 1984; Goetze *et al.*, 1985; Rouzer and Samuelsson, 1985; Soberman *et al.*, 1985a). The 5-lipoxygenase exhibits a requirement for calcium ion and a substrate specificity that differs from that of mammalian ω-6-lipoxygenases (15-arachidonyl lipoxygenase) (Narumiya *et al.*, 1981; Soberman *et al.*, 1985a) and from the potato lipoxygenase (Shimizu *et al.*, 1984), which produces 5-HPETE from arachidonic acid. Only the 5-lipoxygenases from mammalian cells have 5,8,11,14,17-EPA and arachidonic acid as preferred substrates. Removal of the initial double bond decreases the relative substrate activity of 8,11,14-eicosatetraenoic acid by almost 100% relative to arachidonic acid for the 5-lipoxygenase in human PMN (Soberman *et al.*, 1985a) and by 70% in the guinea pig peritoneal PMN 5-lipoxygenase (Ochi *et al.*, 1983). This is quite distinct from the potato lipoxygenase, for which linoleic acid is the preferred substrate, and whose substrate specificity resembles that of human ω-6- or 15-arachidonyl lipoxygenase (Jakschik and Lee, 1980) and soybean lipoxygenase (Hamberg and Samuelsson, 1967), both of which prefer selected C_{18} unsaturated fatty acids. Furthermore, the mammalian 5-lipoxygenases "read" the substrate from the carboxyl end, whereas the mammalian and plant 12- and ω-6-lipoxygenases "read" from the terminal methyl group (Nugteren, 1975; Bach *et al.*, 1984). Thus, 4,7,10,13,16,19-DCHA, which has no Δ5 double bond, is a poor substrate for human, guinea pig, and RBL-1 (Jakschick and Lee, 1980; Ochi *et al.*, 1983; Soberman *et al.*, 1985a) 5-lipoxygenase. The 5-lipoxygenase attacks the carbon at the carboxyl end of the 5,6-double bond of arachidonic acid or EPA, whereas the 12- and ω-6-lipoxygenases act at the ω-carbon of the 14,15- and 11,12-double bond, respectively.

The mammalian 5-lipoxygenase has a pH optimum of 7.5 when obtained from RBL-1 cells and from human leukocytes (Furukawa *et al.*, 1984; Soberman *et al.*, 1985a) and has an M_r of 73,000 on sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis when purified to homogeneity from the RBL-1 line (Goetze *et al.*, 1985) and an M_r of 80,000 by SDS gel when purified to homogeneity from human PMN (Rouzer and Samuelsson, 1985). The enzyme follows the kinetics characteristic of all lipoxygenases, in that it is both activated and inactivated by its hydroperoxide product (Table I).

B. The Dehydration of 5-HPETE to LTA_4

The lipoxygenase of potato tubers, which produces 5-HPETE from arachidonic acid, also coverts 5-HPETE into the 5*S*,12*R*- and 5*S*,12*S*-6-*trans*-LTB_4

TABLE I
PROPERTIES OF THE ENZYMES OF THE 5-LIPOXYGENASE PATHWAY

Enzyme	Source		K_m (μM)	Cofactor	M_r	pH optimum	Subcellular localization
5-lipoxygenase	Human PMN	(AA)	12.2	Ca^{2+}, ATP	80,000	7.5	Cytosol
	Guinea pig PMN		19	Ca^{2+}, ATP			
	RBL-1			Ca^{2+}, ATP	73,000	7.5	
LTA_4 hydrolase	Human PMN	(LTA_4)	20–30	None	70,000	9.0	Cytosol
LTC_4 synthetase	RBL-1	(LTA_4)	5–10	None	—	7.6	Microsomes
		(GSH)[a]	3,000–6,000				
LTB_4 20-hyroxylase	Human PMN	(LTB_4)	0.22–1.0	NADPH	—	7.5	Microsomes
		(NADPH)[b]	1.0				

[a] GSH, glutathione *S*-transferase.
[b] NADPH, reduced nicotinamide-adenine dinucleotide phosphate.

diastereoisomers and produces these compounds in smaller amounts from arachidonic acid (Shimizu *et al.*, 1984). Because these di-HETE isomers are known to be formed by the nonenzymatic hydrolysis of LTA_4, Shimizu *et al.* (1984) postulated that the 5-lipoxygenase of the potato and probably that in mammalian cells may function as a dehydrase synthesizing LTA_4 from 5-HPETE. This has been confirmed by Rouzer *et al.* (1986), who have shown that highly purified human PMN catalyze this same reaction.

C. LTC_4 SYNTHETASE

The conversion of LTA_4 to LTC_4 is catalyzed by a membrane-bound enzyme (Jakschick *et al.*, 1982; Bach *et al.*, 1984) which resides in the microsomal fraction of the RBL-1 cells and can be solubilized by the use of Triton X-104 detergent (Yoshimoto *et al.*, 1985). The RBL-1 cell also possesses a microsomal isomer of glutathione *S*-transferase which conjugates 1-chloro-2,4-dinitrophenol with glutathione. The detergent-solubilized glutathione *S*-transferase and LTC_4 synthetase can be separated by DEAE ion-exchange chromatography. Kinetic parameters indicate that the K_m of the LTC_4 synthetase for LTA_4 (5 to 10 μM) is essentially the same as that reported for the LTA_4 hydrolase from human PMN (Rådmark *et al.*, 1984). In addition, the K_m for glutathione *S*-transferase of 5 mM is within the range of levels reported for many mammalian cells and tissues (Meister, 1984). Thus, the conversion of LTA_4 to LTC_4 or LTB_4 would probably not be determined by the levels of LTA_4, but rather by the relative quantities of hydrolase and synthetase in a particular cell. Furthermore, the activity of LTC_4 synthetase would be altered by the cellular glutathione *S*-transferase as noted by Rouzer and co-workers (1982), who showed an almost linear

decrease in LTC_4 production when compared to glutathione *S*-transferase levels in macrophages phagocytosing zymosan.

D. LTA_4 Hydrolase

LTA_4 hydrolase has been purified to homogeneity from the cytosol of human PMN (Rådmark *et al.*, 1984) and has also been identified in red blood cells (Fitzpatrick *et al.*, 1982) and plasma (Fitzpatrick *et al.*, 1983). It has a molecular size of 70,000 and a broad pH optimum with a peak at 9.0. The apparent K_m of the enzyme for LTA_4 is 20 to 30 μM.

E. LTB_4 20-Hydroxylase

LTB_4 is metabolized to LTB_4-20-hydroxylase (20-OH LTB_4) by an enzyme present in human PMN (Jubiz *et al.*, 1982; Powell, 1984; Shak and Goldstein, 1984a). The monooxygenation at the C_{20} carbon is characteristic of the cytochrome *P*-450-mediated ω-oxidation of PGE_1 and PGA_2 (Powell, 1978, 1979; Okita *et al.*, 1981; Williams *et al.*, 1984) and of the C_{12} oxygenation of lauric acid (Okita *et al.*, 1981) by unique isomers of cytochrome *P*-450. Inhibition of the ω-oxidation of LTB_4 by carbon monoxide (Sumamoto *et al.*, 1984; Shak and Goldstein, 1984b) and modified fatty acids with terminal acetylenic groups (Shak *et al.*, 1985) support cytochrome *P*-450 as catalyzing this reaction. The enzyme resides in the microsomal membrane of these cells (Shak *et al.*, 1985; Soberman *et al.*, 1985b). The conversion of LTB_4 to 20-OH LTB_4 is NADPH dependent. The K_m values for LTB_4 and NADPH are 0.22 and 1.0 μM, respectively, indicating that the rate-limiting step for 20-OH LTB_4 generation is the production of LTB_4 itself since the concentration of NADPH in PMN (50 to 100 μM) is not rate limiting. The conversion of 20-OH LTB_4 to the 20-COOH LTB_4 occurs in the cytosol and the dehydrogenases involved have not been delineated.

F. γ-Glutamyl Transpeptidase and Dipeptidase

LTC_4 is converted to LTD_4 by isolated γ-glutamyl transpeptidase (Hammarström *et al.*, 1980; Örning *et al.*, 1980). RBL-1 cells stimulated with calcium ionophore A23187 generate LTD_4 and LTE_4 as well as LTC_4 (Örning *et al.*, 1980), suggesting the presence of a transpeptidase and a dipeptidase in these transformed cells. γ-Glutamyl transpeptidase has been identified on lymphoid cells (Meister *et al.*, 1976), but has not been observed functionally in resident mouse peritoneal macrophages (Rouzer *et al.*, 1982), human monocytes (Williams *et al.*, 1984), or eosinophils (Weller *et al.*, 1983), which respond to ionophore activation with generation of LTC_4 without conversion. In a complex organ such as lung, immunologic generation of LTC_4 is accompanied by the appearance of LTD_4 and LTE_4 (Lewis *et al.*,

1980a; Hammarström, 1981a), most likely due to the processing of LTC_4 after its release from mast cells. Under physiologic conditions at pH 7.4 in the presence of 50 μM glutamate or leucine, more than 50% of the metabolism of glutathione occurs via transpeptidation rather than hydrolysis (Aellig *et al.*, 1977); thus, if γ-glutamyl transpeptidase is present on cells producing LTC_4, it is possible that the presence of a glutamic acid acceptor in the extracellular medium would facilitate the conversion of LTC_4 to LTD_4. The conversion of LTD_4 to LTE_4 is catalyzed by intact human PMN (Lee *et al.*, 1982), by kidney and liver homogenates (Hammarström, 1981b), and by human plasma. In human PMN this enzyme is a dipeptidase which is localized in the specific granules and released in physiologic calcium ion containing buffers (Lee *et al.*, 1982).

IV. Cellular Specificity of Leukotriene Generation in Response to the Calcium Ionophore and Transmembrane Stimuli

The capacity to metabolize arachidonic acid to product(s) of the 5-lipoxygenase pathway exhibits cellular specificity. PMN generate about 50 ng $LTB_4/10^6$ cells and only minimal amounts of LTC_4 in response to activation with calcium ionophore A23187 (Lee *et al.*, 1983), whereas the quantities and ratios are approximately reversed with ionophore activation of normal eosinophils (Weller *et al.*, 1983); it is likely that the minor product for each cell type is due to cross-contamination. Peripheral blood monocytes produce about 70 ng LTB_4 and 20 ng $LTC_4/10^6$ cells (Williams *et al.*, 1984), whereas adherent alveolar macrophages exhibit augmented generation of LTB_4 in an average 20-fold excess relative to LTC_4 (Fels *et al.*, 1982; Martin *et al.*, 1984). The quantities of leukotriene product generated by each of these cells in response to transmembrane (physiologic) stimuli are substantially less than those obtained with ionophore activation. Pulmonary alveolar macrophages activated by unopsonized or opsonized zymosan and peripheral blood monocyte monolayers ingesting unopsonized zymosan elaborate about one-fourth and one-sixth, respectively, of the quantities of leukotrienes produced by the calcium ionophore (Williams *et al.*, 1984). Furthermore, comparable phagocytosis of particles via monocyte IgG Fc receptors causes minimal LTB_4 and LTC_4 generation, as compared to stimulation by zymosan acting at its specific β-glucan recognition unit (Williams *et al.*, 1984; Czop and Austen, 1985), indicating stimulus-specific membrane responses for leukotriene generation.

N-Formylated peptides mediate activation of the 5-lipoxygenase pathway in human PMN as assessed by the identification of 5-HETE, LTB_4, and the ω-oxidation products of LTB_4 (Bokoch and Reed, 1980; Clancey *et al.*, 1983). In one of these studies, activation of the 5-lipoxygenase pathway by *N*-

formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) was recognized only after the introduction of exogenous arachidonic acid to provide substrate, suggesting that FMLP activated the 5-lipoxygenase but not the phospholipase A_2 (Clancey *et al.*, 1983). FMLP also initiated the generation of immunoreactive sulfidopeptide leukotrienes and LTB_4 in a dose-dependent manner from monocyte monolayers that had been pretreated with cytochalasin B (Williams *et al.*, 1986). The sum of the two classes of leukotrienes generated was about one-sixth that obtained from monocytes stimulated with the calcium ionophore A23187. The requirement for cytochalasin B in order for FMLP, but not the calcium ionophore, to stimulate leukotriene generation is compatible with the ability of cytochalasin B to augment certain stimulus-specific transmembrane responses (Wessels *et al.*, 1971) that are not dependent on the integrity of the cytoskeleton.

After stimulation of PMN with unopsonized zymosan, 5-HETE and 6-*trans*-LTB_4 diastereoisomers remain cell associated, LTB_4 is only partially released, and the ω-oxidation products of LTB_4 are preferentially extracellular (Williams *et al.*, 1985). In contrast, with ionophore stimulation, only 5-HETE has any duration of intracellular residence; 6-*trans*-LTB_4 diastereoisomers, LTB_4, and the ω-metabolites of LTB_4 are largely rapidly released. In contrast, monocytes in monolayers stimulated with zymosan do not retain LTB_4 intracellularly (Williams *et al.*, 1984). This difference between PMN and monocytes in monolayer may be related to trapping of product in the phagolysosome of the PMN suspensions.

Mouse bone marrow-derived, interleukin-3-dependent mast cells generate predominantly LTC_4 with some LTB_4 and minimal PGD_2 in response to activation with either calcium ionophore or antigen challenge after sensitization with IgE (Razin *et al.*, 1983, 1984; Stevens *et al.*, 1985). Although total product generation by perturbation of the IgE Fc receptor is only about one-sixth that obtained with ionophore, the generation during the first 5 minutes of activation by the immunologic stimulus, which then plateaus, is greater than for the ionophore.

V. Biologic Effects of the Leukotrienes

A. Sulfidopeptide Leukotrienes

Sulfidopeptide leukotrienes LTC_4, LTD_4, and LTE_4 comprise the activity previously recognized as slow reacting substance of anaphylaxis (SRS-A) and are potent spasmogenic agents on nonvascular smooth muscle (Drazen *et al.*, 1980). LTC_4 and LTD_4 evoke pulmonary mechanical bronchoconstrictor responses after intravenous infusion in guinea pigs (Leitch *et al.*, 1983a) and after inhalation challenge in both guinea pigs (Leitch *et al.*, 1983b) and

humans (Holroyde *et al.*, 1981; Weiss *et al.*, 1982, 1983; Griffin *et al.*, 1983; Barnes *et al.*, 1985; Smith *et al.*, 1985). In normal humans, the sulfidopeptide leukotrienes are relatively less active on central airways as assessed by the forced expiratory volume at 1 second (FEV_1) compared to their potency in compromising peripheral airways as assessed by expiration at 30% of vital capacity ($\dot{V}_{30}$) (Weiss *et al.*, 1982, 1983). Griffin and co-workers (1983) reported that the airway responsiveness of six asymptomatic asthmatic subjects to inhaled LTD_4 as assessed by $\dot{V}_{30}$ was only slightly greater than that of normal subjects, even though the asthmatic subjects had the expected hyperresponsiveness to inhaled histamine (Griffin *et al.*, 1983) for this measurement and by the FEV_1. Smith *et al.* (1985) found that LTD_4 had an equal effect on both large and small airways, as evidenced by the measurements of the changes in specific conductance and $\dot{V}_{30}$, and both asthmatic and normal subjects had the same degree of increased reactivity to LTD_4 as to methacholine inhalation, but the response of their normal population required relatively more LTD_4 than that noted by others.

The cumulative experience with inhalation not only of LTD_4 (Griffin *et al.*, 1983; Weiss *et al.*, 1983) but also of LTC_4 (Weiss *et al.*, 1982) and LTE_4 indicates a preferential action on the peripheral airways of normal subjects as assessed by the decrement in $\dot{V}_{30}$ by 30% or more without an appreciable fall in the FEV_1. By this measurement the asthmatic patients are relatively less hyperresponsive to the sulfidopeptide leukotrienes than to other agonists (Griffin *et al.*, 1983). However, the asthmatic patients are markedly hyperreactive to sulfidopeptide leukotrienes as compared to normal individuals in terms of the elicited decrease in FEV_1, suggesting that the lesion of asthma is central rather than peripheral airways "hyperreactivity." The latter may reflect not only constriction but also permeability changes with edema.

Bronchial mucosal explants respond in tissue culture by enhanced mucus secretion to the presence of as little as 10^{-9} *M* LTC_4 or LTD_4 (Marom *et al.*, 1982), but the response is not stereospecific and thus is not likely to be receptor mediated (Coles *et al.*, 1983). Augmented postcapillary venular permeability was shown for dermal vascular beds of the guinea pig responding to locally injected LTC_4, LTD_4, and LTE_4 by leakage of intravenously administered dye (Drazen *et al.*, 1980; Lewis *et al.*, 1980b), and by the leakage of intravascular fluorescent dye into the tissue of the hamster cheek pouch after topical application of each leukotriene (Dahlén *et al.*, 1981). The intradermal administration of LTC_4, LTD_4, and LTE_4 to normal human subjects produced a local wheal and flare response, in which the wheal, representing enhanced venopermeability, was sustained for 2 to 4 hours (Camp *et al.*, 1983; Soter *et al.*, 1983).

The capacity of LTC_4 and, to a lesser extent, of LTD_4 to constrict arterioles was initially demonstrated in guinea pig skin at the site of intrader-

mal administration of the leukotriene (Drazen *et al.*, 1980; Lewis *et al.*, 1980b), and was confirmed by the response to topical administration of the hamster cheek pouch (Dahlén *et al.*, 1981) and by the blanching of the elicited wheal in normal human skin (Camp *et al.*, 1983; Soter *et al.*, 1983). Coronary vasoconstriction in response to these molecules has been shown after a direct infusion into a major coronary vessel of the sheep *in vivo* (Michelassi *et al.*, 1982) and after infusion into the systemic venous circulation of the anesthetized rat (Pfeffer *et al.*, 1983). In each case, myocardial contractility was compromised on a dose-dependent basis due to coronary arterial contraction. The *in vivo* administration of LTC_4 by intravenous infusion to the anesthesized rat also evoked renal vasoconstriction (Badr *et al.*, 1984), and increased systemic vasopermeability, thus compromising renal perfusion as well as cardiac output by two mechanisms. LTC_4 administered into the right atrium of normal human subjects caused a transient marked vasodilation in the systemic and possibly pulmonary circulation (Kaijser, 1982).

B. Leukotriene B_4

The biologic activities of LTB_4 demonstrated *in vitro* include chemotaxis of human PMN (Ford-Hutchinson *et al.*, 1980), eosinophils (Nagy *et al.*, 1982), and presumptively mixed T lymphocytes (Payan and Goetzl, 1981), chemokinesis of monocytes (Palmer *et al.*, 1980), aggregation of PMN (Ford-Hutchinson *et al.*, 1980), enhanced expression of C3b receptors on PMN and eosinophils (Nagy *et al.*, 1982), release of lysosomal enzymes from PMN (Showell *et al.*, 1982), and augmentation of PMN adherence to endothelial cell monolayers by a selective effect on the endothelial cells (Hoover *et al.*, 1984). *In vivo* intradermal injection of LTB_4 promoted PMN infiltration into the skin of rhesus monkeys (Lewis *et al.*, 1981) and humans (Camp *et al.*, 1983; Soter *et al.*, 1983); the lesion is characterized by induration and tenderness, which are most prominent 4 to 6 hours after injection. LTB_4 is also spasmogenic for airway smooth muscle. However, unlike the SRS-A components, which are directly spasmogenic for bronchial tissue *in vitro*, LTB_4 evokes the response indirectly, via stimulated biosynthesis of constrictor cyclooxygenase products (Sirois *et al.*, 1981). LTB_4 is also immunoregulatory, inducing increased numbers of suppressor T lymphocytes from precursors (Atluru and Goodwin, 1986) and the inhibition of mitogen-induced T cell proliferation (Rola-Plezczynski *et al.*, 1982; Payan and Goetzl, 1983) and immunoglobulin synthesis (Payan and Goetzl, 1983).

VI. Receptor-Dependent Mediation of Leukotriene Effects

The substantial evidence that the sulfidopeptide leukotrienes exert their physiologic effects through distinct receptors is based upon stereochemical

requirements of the agonist and saturable binding of radioligand but has not progressed to a correlation of binding with function or to receptor characterization. Chemical analogs of the sulfidopeptide leukotrienes of the LTC_4 and LTD_4 subclasses demonstrate stereospecificity for their agonist action with strict requirements for the stereochemistry at C-5 and C-6 of the eicosanoid backbone to which the hydroxyl and sulfidopeptide domains are attached, respectively. That there were subclass-specific receptors for LTC_4 and LTD_4 was suggested initially by functional studies. The molar concentrations of LTC_4, LTD_4, and LTE_4 that are required to elicit the same magnitude of contractile response are in the respective ratio of 4:1:5 in guinea pig ileum, 30:1:100 in guinea pig lung parenchymal strips, and 1:1:0.1 in guinea pig tracheal spirals (Drazen *et al.*, 1983). Contraction of guinea pig ileal smooth muscle by LTC_4 requires a 60-second latent period after exposure of the tissue to the agonist, whereas the constriction elicited by LTD_4 is immediate (Krilis *et al.*, 1983a). LTC_4 and LTE_4 elicit a monophasic contraction of guinea pig parenchymal strips, whereas LTD_4 elicits a biphasic response (Drazen *et al.*, 1980). The contractile reaction to low doses of LTD_4 is inhibited by FPL 55712, but the spasmogenic response to LTD_4 at concentrations in excess of 10^{-10} *M* and to any concentration of LTC_4 is not affected by FPL 55712 (Drazen *et al.*, 1980). Recent studies have documented that the LTC_4-induced and high-dose LTD_4-induced contractions of parenchymal strips are inhibited by diltiazem, whereas the low-dose phase of LTD_4-induced constriction is unaffected by the calcium antagonist (Drazen *et al.*, 1984).

Radioligand binding studies, which have included assessment for saturation, reversibility and competition with unlabeled homoligand and heteroligand, have also indicated that the LTC_4 binding is distinct from that for LTD_4 (Hogaboom *et al.*, 1983; Krilis *et al.*, 1983b, 1984; Pong and DeHaven, 1983; Pong *et al.*, 1983; Sun *et al.*, 1986). The equilibrium dissociation constant (K_d) for LTC_4 binding is 5 to 8 n*M* (Hogaboom *et al.*, 1983; Krilis *et al.*, 1983b, 1984; Pong *et al.*, 1983; Sun *et al.*, 1986), and for LTD_4 is ~0.2 n*M* (Pong and DeHaven, 1983). LTE_4 competes for the LTD_4 binding unit in guinea pig lung but not effectively for that for LTC_4 binding, suggesting that the expression of LTE_4 activity may be through the LTD_4 receptor (Pong and DeHaven, 1983). However, in the presence of 1.3 n*M* calcium, LTE_4, LTE_4 sulfoxide, and LTE_5 enhanced the contraction of tracheal spirals to histamine in a time- and dose-dependent fashion (Lee *et al.*, 1984a, 1985c), whereas LTC_4, LTD_4, LTE_4-C-1 monoamide, LTE_4 cys-amide, and *N*-acetyl-LTE_4, at concentrations that produced the same constriction as LTE_4, did not augment histamine responsiveness of tracheal spirals (Lee *et al.*, 1984a). LTD_4 augmented histamine responsiveness of tracheal smooth muscle only in the presence of very low calcium concentrations (0.1 m*M*) (Creese and Bach, 1983). These findings indicate that LTE_4 and its oxidative

metabolite induce central airway hyperresponsiveness to histamine *in vitro* by a mechanism that is critically dependent on the integrity of the eicosanoid carboxyl group at C-1, the cysteine carboxyl group, and the free NH_2-terminal amino group of the cysteine adduct in the structure of LTE_4. The dissociation of the contractile response from the enhancement of histamine responsiveness, and the strict requirements for structure–function, suggest that the latter may be mediated through a recognition unit distinct from the shared receptor with LTD_4 which mediates constriction.

The ease with which LTC_4 radioligand binding was observed in diverse tissue sonicates and their fractions, irrespective of whether the tissue was known to engage in a functional response, prompted a search for binding activity in rat liver. The cytosol exhibited binding with a K_d of about 1 n*M* which was competitively blocked by LTC_4 but was only poorly prevented by LTD_4, LTE_4, and LTB_4. The [^{3}H]LTC_4 binding activity was isolated chromatographically and was identified as a 23,000 M_r subunit of glutathione *S*-transferase, termed Ya or ligandin, by function, immuno-cross-reactivity, and photoaffinity labeling. Although LTD_4 radioligand binding does appear to occur via a trans-membrane-linked receptor (Mong *et al.*, 1985), the putative receptor for the inherent spasmogenic activity of LTC_4 that occurs without conversion to LTD_4 (Drazen *et al.*, 1980; Krilis *et al.*, 1983a) is not defined. The high-affinity, stereospecific binding of [^{3}H]LTC_4 to cytosolic and microsomal glutathione-*S*-transferase (Sun *et al.*, 1986) accounts for the radioligand binding unrelated to a defined tissue response, may reveal a further function of this family of isoenzymes, and indicates a binding function that must be excluded in seeking the putative spasmogenic receptor.

Radioligand binding studies suggest the existence of a nonhomogeneous population of LTB_4 binding sites on the human PMN on the basis of a biphasic [^{3}H]LTB_4 dissociation curve that is seen when excess unlabeled LTB_4 is added to the cells. The K_d of the high-affinity receptor was ~0.5 n*M* and the K_d of the low-affinity receptor was ~300 n*M* (Goldman and Goetzl, 1982). The high-affinity receptor is believed preferentially to mediate chemotaxis, and the low-affinity receptor lysosomal enzyme release (Goldman *et al.*, 1983).

VII. Modulation of Arachidonic Acid Metabolism by the Introduction of Dietary Alternative Fatty Acids

On the basis of accumulated understanding of the enzymatic pathways, cellular specificity, and biologic functions of each leukotriene, at least some of which are mediated by defined specific receptors, it is appropriate to attempt *in vivo* regulation of leukotriene proinflammatory effects. The introduction of marine-derived dietary fatty acids modulates some steps in arach-

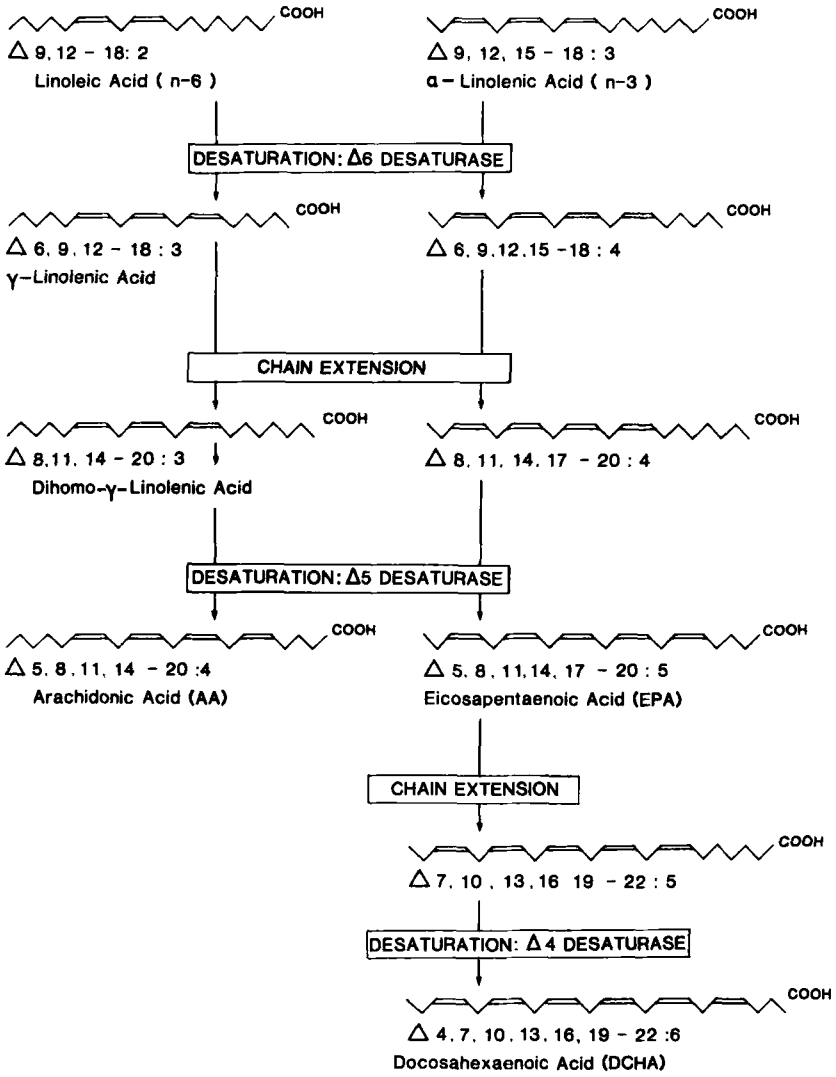

FIG. 3. Desaturation and chain elongation of N-3 and N-6 fatty acids to form arachidonic, eicosapentaenoic, and docosahexaenoic acids.

idonic acid metabolism and yields some attenuated analogs of the tetraene mediators.

EPA and its natural hydrocarbon chain extension and desaturation product DCHA are members of the linolenic acid (18:3, N-3) family, and are prominent in green leaves and especially in marine lipids (Fig. 3). These

fatty acids are termed N-3 fatty acids to reflect the position of the double bond furthest from the carboxylic acid. Arachidonic acid is a member of the linoleic acid (18:2, N-6) family and predominates in terrestrial animals and in most plant seeds (Fig. 3). N-3 fatty acids are incorporated into the tissue phospholipids of humans and other mammals who consume a predominantly fish oil-enriched diet. Crawford (1983) suggested that there may be two sources of eicosanoid precursor fatty acids: a metabolic pool, which is closely related to current dietary intake of preformed eicosanoid precursor fatty acids, and a cell membrane pool which is released after cell activation and is linked to dietary supply and metabolism of precursor fatty acids. This view is supported by the demonstration that dietary supplementation with linoleic acid for 24 hours led to a 7-fold increase in urinary prostaglandin excretion without changing membrane phospholipid fatty acid profiles in essential fatty acid-deficient rats (Ramestra *et al.*, 1986). During long-term dietary N-3 fatty acid supplementation, EPA is not incorporated into phosphatidylinositol or phosphatidylserine of human platelets *in vivo* and is not detectable in phosphatidic acid upon stimulation with thrombin (Schacky *et al.*, 1986). However, EPA is released from platelet phospholipids such as phosphatidylcholine and is metabolized to thromboxane B_3. In contrast, platelets incorporate [^{3}H]EPA into phosphatidylinositol *in vitro* and after platelet stimulation [^{14}C]EPA appears in phosphatidic acid as free fatty acid, and is transformed to ^{14}C-labeled thromboxane B_3. These findings suggest that the fatty acid compositions of platelet phospholipid subclasses are regulated with a high degree of specificity *in vivo* and that qualitative differences exist between *in vivo* and *in vitro* uptake of EPA into platelet membrane phospholipids. Presumably, EPA is released by the action of a phospholipase A_2 after *in vivo* incorporation and, in addition, by the combined action of a phospholipase C and a diacylglycerol phospholipase/kinase enzyme after *in vitro* incorporation.

EPA and DCHA competitively inhibit the conversion of arachidonic acid to cyclooxygenase products and, to the extent that they are formed, the prostaglandin endoperoxide and thromboxane A_3 derived from EPA are substantially less active than their arachidonic acid-derived counterparts in eliciting aggregation of human platelets (Dyerberg *et al.*, 1978; Whitaker *et al.*, 1979). In contrast, neither EPA nor DCHA is an inhibitor of 5-lipoxygenase, and with respect to the oxidative metabolism of EPA and DCHA by the 5-lipoxygenase cascade, the ratio of substrate specificity of arachidonic acid:EPA:DCHA for cell-free preparations of 5-lipoxygenase from guinea pig and human PMN is 100:125:1 (Ochi *et al.*, 1983; Soberman *et al.*, 1985a). Whereas EPA is converted sequentially by the specific enzymes of the 5-lipoxygenase pathway to the fully bioactive sulfidopeptide leukotrienes LTC_5, LTD_5, and LTE_5, and to LTB_5 which has attenuated function (Ham-

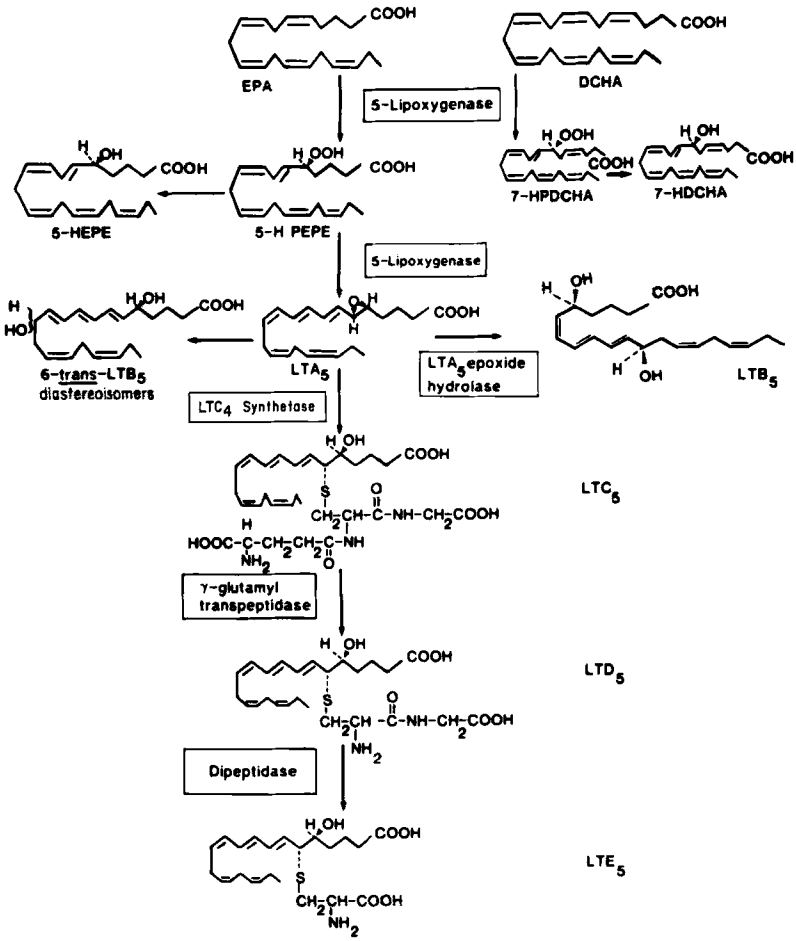

FIG. 4. Metabolism of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DCHA) by the 5-lipoxygenase pathway.

marström, 1980, 1981a; Murphy *et al.*, 1981; Dahlén *et al.*, 1982; Lee *et al.*, 1984c; Leitch *et al.*, 1984; Terano *et al.*, 1984a,b) (Fig. 4), DCHA is metabolized only to 7- and 4-hydroperoxy DCHA and their reduction products, 7- and 4-hydroxy DCHA, respectively (Lee *et al.*, 1984d).

VIII. Biologic Properties of EPA and DCHA-Derived 5-Lipoxygenase Metabolites

LTB_5, the 5,12-dihydroxy derivative of EPA analogous to LTB_4, is substantially less active than LTB_4 in a number of proinflammatory functions:

LTB_5 has from 3 to 10% of the activity of LTB_4 in eliciting PMN chemotaxis (Goldman *et al.*, 1983; Lee *et al.*, 1984c,d; Leitch *et al.*, 1984; Terano *et al.*, 1984a), chemokinesis (Terano *et al.*, 1984a), and aggregation (Lee *et al.*, 1984c), and in augmentation of bradykinin-induced vasopermeability (Terano *et al.*, 1984a). LTB_5 is not only less potent than LTB_4 in eliciting PMN chemotaxis and aggregation, but is also not capable of eliciting the maximum response produced by LTB_4, indicating that it is a weak and partial agonist relative to LTB_4 (Lee *et al.*, 1984c). LTC_4 and LTC_5 are equipotent at constricting parenchymal strips and are each 4 orders of magnitude more active than LTB_4 as contractile agonists (Dahlén *et al.*, 1982; Leitch *et al.*, 1984). 5-Hydroxy-EPA (5-HEPE) and 7-hydroxy-DCHA (7-HDCHA) are inactive as chemotactic agents for human PMN, whereas 5-HETE demonstrates 1 to 2% of the chemotactic activity of LTB_4 (Lee *et al.*, 1984c).

IX. Effects of EPA and DCHA in Animal Studies

A. Dietary Incorporation of Alternative Fatty Acids and Generation of 5-Lipoxygenase Pathway Products *ex Vivo* or *in Vivo*

Murine mastocytoma cells grown in the peritoneal cavities of mice fed an EPA-rich diet have ~50% of their phospholipid arachidonic acid replaced by EPA (Murphy *et al.*, 1981). When these cells were removed and challenged *in vitro* with ionophore A23187, they generated LTB_4, LTB_5, LTC_4, and LTC_5 as defined by RP-HPLC and integrated optical density. There was a 12-fold reduction of LTC (LTC_4 and LTC_5) and a 17-fold reduction of LTB (LTB_4 and LTB_5) generation when compared with mastocytoma cells grown in mice on a control diet, suggesting inhibition of the 5-lipoxygenase pathway. LTB_4 and LTB_5 were generated in approximate proportion to the tumor content of arachidonic acid and EPA, respectively, whereas LTC_4 was preferentially elaborated relative to LTC_5 with an LTC_4:LTC_5 ratio of 10:1.

The effect of a fish oil-enriched diet on 5-lipoxygenase-derived mediator generation in response to an immune complex reaction in the peritoneal cavities of rats was not inhibitory of total product generation as assessed by radioimmunoassay after RP-HPLC (Leitch *et al.*, 1984). Rats on the fish oil-enriched diet incorporated EPA into pulmonary and splenic tissues with an EPA:arachidonic acid ratio of 2:1, whereas the rats on the beef fat diet showed no detectable EPA. The total quantities of immunoreactive sulfidopeptide and dihydroxy leukotrienes generated in the peritoneal cavities of rats on the fish oil-enriched diet were similar to those generated in rats on the beef diet and for rats on the fish oil supplement included the pentaene

products LTC_5, LTD_5, LTE_5, and LTB_5. There was a 2- to 7-fold preferential generation of immunoreactive LTB_5 over LTB_4 in the fish oil-enriched diet rats, as compared to a ratio of <1 for the pentaene to tetraene sulfidopeptide leukotrienes. The finding that the fish oil-enriched diet favors the immunologic generation of LTB_5, which has attenuated biologic activity when compared to LTB_4, suggests that EPA-enriched tissues may produce less proinflammatory activity than tissues that are EPA poor.

Supplementation of a normal rat diet with 240 mg/kg/day of the ethyl ester of EPA (75% pure EPA) for 4 weeks caused a significant increase in the EPA content of peritoneal leukocyte phospholipids without changing the arachidonic acid or linoleic acid content. The EPA:arachidonic acid ratios in the EPA-fed and control animals were 0.1 and 0.03, respectively, with the EPA in the control group being introduced in the standard rat chow (Terano *et al.*, 1984b). The total quantities of LTB generated by ionophore activation of leukocytes, as determined by RP-HPLC and integrated UV absorbance in the two groups of rats, were similar, but the production of LTB_4 was less in the EPA-fed animals as observed for the entirely *in vivo* study with rats (Leitch *et al.*, 1984). The ratio of EPA:arachidonic acid in the leukocytes correlated with the LTB_5:LTB_4 ratios produced after leukocyte stimulation as determined earlier for the *ex vivo* analysis of the mastocytoma cells (Murphy *et al.*, 1981).

B. *In Vivo* Incorporation of Alternative Fatty Acids and Pharmacologic Effects

The possible modulating influence of fish oil diets on models of inflammatory disease has been evaluated in two murine models for systemic lupus erythematosus (NZB-NZW/F_1 and MRL/MP-*lpr*) (Prickett *et al.*, 1981; Kelley *et al.*, 1985) and a rat model for rheumatoid arthritis (Type II collagen-induced arthritis) (Prickett *et al.*, 1984). Fish oil-enriched diets inhibited the severity of glomerulonephritis in the lupus erythematosus models (Prickett *et al.*, 1981; Kelley *et al.*, 1985; Robinson *et al.*, 1986) but increased the incidence of Type II collagen-induced arthritis in rats (Prickett *et al.*, 1984). The protective effects of 5 and 25% menhaden oil diets on renal disease in the NZB-NZW mice differed incrementally, whereas the content of tissue N-3 fatty acids was virtually identical (Robinson *et al.*, 1986). Furthermore, diets containing 2 and 6.2% EPA (in the form of 75% EPA ethyl ester) conferred no protective effects on renal disease in the NZB-NZW mice, although the tissue levels of EPA and DCHA were similar to those in mice protected with 25% menhaden oil. Thus, protection from nephritis in the NZB-NZW mice receiving fish products in the form of menhaden oil did not correlate with the tissue content of N-3 fatty acids (Robinson *et al.*, 1986).

In the MRL/MP-*lpr* model of fulminant systemic lupus erythematosus

(Kelley *et al.*, 1985), a 10% EPA ethyl ester diet decreased lymphoid hyperplasia, prevented an increase in macrophage surface Ia expression, reduced the formation of circulating immune complexes, delayed the onset of renal disease, and prolonged survival. In addition, an EPA ethyl ester-enriched diet decreased the *ex vivo* inherent generation of cyclooxygenase pathway products by the kidney, lung, and peritoneal macrophages.

The effects of a menhaden oil-enriched diet on immediate hypersensitivity as assessed by pulmonary mechanical responses to antigen challenge were evaluated in passively sensitized and anesthetized guinea pigs pretreated with mepyramine to uncover the leukotriene and prostaglandin contributions (Lee *et al.*, 1985a). After 9 weeks the EPA:arachidonic acid ratio of the pulmonary tissue in animals on the menhaden oil-enriched diet was 2:1, whereas EPA was not detectable in the animals on the beef fat diet. Antigen challenge in both groups of animals elicited a decrease in dynamic compliance (C_{dyn}) and pulmonary conductance (GL), with the decrement in C_{dyn} being significantly greater in the animals on the fish oil-enriched diet. Inasmuch as EPA and DCHA inhibit the cyclooxygenase pathway, these results are consistent with the views that the tissue levels of alternative fatty acids either partially inhibit the generation of bronchodilator prostaglandins released after antigen challenge and/or divert both EPA and arachidonic acid metabolism from the cyclooxygenase to the 5-lipoxygenase pathway, resulting in the generation of more bronchoconstrictor sulfidopeptide leukotrienes. The finding that indomethacin pretreatment enhanced the impairment of pulmonary function in beef fat-fed animals after antigen challenge, but had no significant effect on the decrements in pulmonary mechanics in fish oil-fed animals, indicates the *in vivo* inhibition of the cyclooxygenase pathway in the latter group.

When the changes in arterial plasma concentrations of immunoreactive LTB_4 were compared in the two groups of guinea pigs after antigen challenge, the time-dependent increase in plasma LTB was significantly greater in the beef fat-fed animals (Lee *et al.*, 1984b). The combination of indomethacin and mepyramine pretreatment of the animals markedly augmented the antigen-induced increase in arterial plasma immunoreactive LTB_4 concentrations in fish oil-fed guinea pigs but had no effect on LTB (LTB_4 + LTB_5) levels in the fish oil-fed animals. The failure of immunoreactive thromboxane B levels to rise in fish oil-fed animals and its marked increase in beef fat-fed animals undergoing anaphylaxis provide direct evidence for blockage of the cyclooxygenase pathway in the former group (Lee *et al.*, 1986). The measurements of arterial plasma LTB indicate that indomethacin treatment alone or a menhaden oil-enriched diet alone augment the metabolism of arachidonic acid by the 5-lipoxygenase pathway during anti-

gen-induced anaphylaxis in the mepyramine-pretreated guinea pig model (Lee *et al.*, 1985a, 1986).

In contrast, when the antigen was delivered by inhalation, the fish oil-enriched diet attenuated the decrements in pulmonary mechanics after challenge (Israel *et al.*, 1980). The protective effects of a fish oil-enriched diet against the bronchoconstrictor response in antigen inhalation challenge, which may more closely resemble extrinsic human asthma, may be related to the inhibition of mediator generation from cells that are activated during inhalation antigen challenge, but not during intravenous antigen challenge.

Pretreatment of rats with a cyclooxygenase inhibitor, ibuprofen, enhanced survival during endotoxic shock (Wise *et al.*, 1980), suggesting that suppression of the generation of prostanoid metabolites may decrease mortality. The morbidity of shock in a menhaden oil-fed group of rats was significantly delayed as compared to a safflower oil-fed group after intravenous endotoxic challenge. The reduced mortality in the menhaden oil-fed animals was associated with a decreased capacity of white blood cells to generate thromboxane B_2 in response to stimulation with collagen (Mascioli *et al.*, 1985).

The animal experiments with a fish oil-enriched diet illustrate contrasting effects in different models such as nephritis in mice (NZB-NZW/F_1 or MRL/MP-*lpr*), as compared to Type II collagen arthritis in rats and opposing actions on pulmonary function in sensitized guinea pigs subjected to antigen challenge by different routes (intravenous versus aerosol). The outcome thus depends upon the cells involved in preparing the animal for the pathologic immune response and/or on the balance of active and attenuated products of the cyclooxygenase and 5-lipoxygenase products elicited by the response. Appreciation of this diversity will be critical in evaluating epidemiologic data at the human level of disease prevention and especially in planning any interventions in established pathobiologic states.

X. Effects of EPA and DCHA in Human Studies

A. *In Vitro* Effects of Nonesterified EPA and DCHA on the 5-Lipoxygenase Pathway

The capacity of nonesterified EPA and DCHA, respectively, to modulate the oxidative metabolism of membrane phospholipid-derived arachidonic acid by the 5-lipoxygenase pathway in human PMN was quite different (Lee *et al.*, 1984d) whereas both inhibit the cyclooxygenase pathway (Needleman *et al.*, 1979; Corey *et al.*, 1983). In these *in vitro* experiments, exogenous fatty acids (5 to 40 μg/ml) did not alter the release of [^{3}H]arachidonic acid from ionophore-activated [^{3}H]arachidonic acid-labeled PMN. The metabo-

lism of low concentrations of exogenous EPA (5 and 10 μg/ml) not only generated EPA-derived products, but also stimulated the formation of 5-HETE and 6-*trans*-LTB_4 diastereoisomers, which serve as markers for 5-HPETE and LTA_4, from membrane-derived arachidonic acid. LTB_4 production was diminished throughout the EPA dose–response, beginning at 5 μg/ml EPA and reaching 50% suppression at 10 μg/ml and 84% suppression at 40 μg/ml. DCHA did not stimulate the metabolism of membrane-derived arachidonic acid, did not inhibit LTB_4 generation, and was not a substrate for leukotriene formation. Thus, in contrast to DCHA, EPA attenuated the generation of LTB_4 (Lee *et al.*, 1984d; Prescott, 1984) and was converted to LTB_5.

B. *In Vivo* Incorporation of Alternative Fatty Acids

Dietary enrichment with 3.2 g EPA and 2.2 g DCHA daily for 6 weeks in 7 normal subjects resulted in a greater than 7-fold increase in the EPA content of peripheral blood PMN and monocytes (Lee *et al.*, 1985b), respectively, without a change in DCHA in either cell type. EPA was not detectable in PMN and monocyte phospholipids before the dietary supplementation, whereas the EPA:arachidonic acid ratios at 6 weeks were 1:8 and 1:6 for PMN and monocytes, respectively. As assessed by the response to activation with the calcium ionophore A23187, the incorporation of EPA into PMN at 6 weeks was accompanied by a reduction in the release of [^{3}H]arachidonic acid from labeled cells and a suppression in the maximum generation of 3 products of the 5-lipoxygenase pathway, 5-HETE, 6-*trans*-LTB_4 diastereoisomers, and LTB_4. At the same time period there was a reduction in the ionophore-induced release of [^{3}H]arachidonic acid from monocytes in monolayer and a reduction in the generation of LTB_4 and to a lesser extent in 5-HETE without an appreciable diminution in the maximal production of 6-*trans*-LTB_4 diastereoisomers. Pentaene products of the 5-lipoxygenation of EPA, namely, 5-HEPE and LTB_5, were elaborated with ionophore activation of both cell types but their ratio to tetraene products was substantially less than the ratio of EPA:arachidonic acid in each cell type. The inhibition of product generation in PMN was significant for the summed products, 5-HETE + 5-HEPE, 6-*trans*-diastereoisomers of LTB_4 and LTB_5, and LTB_4 + LTB_5, whereas inhibition of the pathway in monocytes at 6 weeks of dietary supplementation was limited to LTB_4 + LTB_5. PMN were completely inhibited in their adherence to LTB_4-primed bovine endothelial cell monolayers and PMN were inhibited in their chemotactic response to LTB_4 by 70% at 6 weeks, as compared to the normal diet period (Lee *et al.*, 1985b). The suppression of PMN and monocyte 5-lipoxygenase pathway metabolism and PMN chemotactic and adherence functions had recovered by 6 weeks after the diet was discontinued.

In a study of 12 patients with severe persistent asthma receiving EPA

ethyl ester daily for 8 weeks, the chemotactic response of PMN to LTB_4, C5a, and FMLP was inhibited by 54 to 73% with a 4.0 g EPA daily supplement but not with a 0.1 g EPA supplement. Monocyte chemotaxis was not affected by either dose of EPA (Wong *et al.*, 1986). Thus, a fish oil-enriched diet in humans impairs the proinflammatory product generation of peripheral blood leukocytes and alters the function of PMN. The duration of dietary supplementation with fish oil appears to be critical for the effect on PMN and this may prove to be even longer for monocytes. Dietary enrichment with fish oil (Fitzerald *et al.*, 1986) for 4 weeks in volunteers led to the generation of LTB_5 from ionophore-stimulated PMN, but no suppression of LTB_4 biosynthesis and no inhibition of the PMN chemotactic response to FMLP. The effects observed in the PMN of normals at 6 to 8 weeks (Lee *et al.*, 1985b) and asthmatics at 8 weeks (Wong *et al.*, 1986) were not apparent at an intermediate time period. This contrasts to the effects of EPA on platelets, since functional impairment of this cell type is evident within the first week of dietary supplementation with EPA. The mechanisms of the EPA effect on leukocytes appear to be both impaired arachidonic acid release from membrane stores and conversion to 5-HPETE (Lee *et al.*, 1985b).

EPA ingestion also affects the distribution of T lymphocyte subsets and T cell proliferative responses *in vitro*. The uptake of [^{3}H]thymidine elicited by phytohemagglutinin (PHA) increased by ~100% after 8 weeks on 0.1 and 4.0 g/day EPA in asthmatic subjects receiving steroids (Wong *et al.*, 1986). The ratio of T suppressor to T helper lymphocytes changed from a prediet mean of 3.1 and 2.3 in subjects fed 0.1 and 4.0 g EPA, respectively, to 3.4 and 1.6, respectively, after EPA. Thus, the increased reactivity to PHA in the high EPA dose group was attributed to an expansion of T suppressor cells at the expense of T helper cells, whereas both subsets shared in the increase in PHA reactivity in patients receiving low doses of EPA.

C. A Comparison of the Effects of Nonesterified and Esterified EPA on Arachidonic Acid Metabolism in Human PMN

A comparison of the effects of nonesterified (Lee *et al.*, 1984d) and esterified EPA (Lee *et al.*, 1985b) on arachidonic acid metabolism in human PMN demonstrates interesting differences. Nonesterified EPA does not alter arachidonic acid release from membrane phospholipids, whereas esterified EPA causes a substantial inhibition of phospholipase A_2. When PMN are activated in the presence of low concentrations of EPA, the 5-lipoxygenase activity is enhanced. This effect is in sharp contrast to that of esterified EPA, which suppresses the 5-lipoxygenase activity. The presence of nonesterified EPA inhibits the epoxide hydrolase, thus selectively suppressing the elaboration of LTB_4. Although there is also inhibition of LTB_4

generation in the presence of esterified EPA, this represents decreased substrate availability due to the combined suppression of endogenous fatty acid hydrolysis and 5-lipoxygenase activity. Since both esterified and non-esterified EPA are likely to exert a regulating influence on the biochemistry and function of leukocytes at the site of an inflammatory response, the antiinflammatory action will depend on the relative concentrations of the free and esterified alternative (EPA) fatty acid and may include an inhibitory effect on phospholipase A_2 and modulating influences on several of the specific enzymes of the cyclooxygenase and lipoxygenase pathways.

XI. Conclusions

The nature and actions of SRS-A were reviewed in *Advances in Immunology* in 1969 by Orange and Austen (1969) just prior to the period in which the partial purification of immunologically generated material allowed presumptive identification of a sulfur content (Orange *et al.*, 1973, 1974; Wasserman and Austen, 1976; Weller *et al.*, 1986) and the demonstration that a product with indistinguishable chromatographic and functional properties could be elaborated by leukemic human basophils upon activation with the calcium ionophore (Lewis *et al.*, 1975). The subsequent production of SRS-A for structure characterization (Murphy *et al.*, 1979) was facilitated by these data. The stereochemical definition based upon total synthesis (Corey *et al.*, 1980), and the delineation of the biosynthetic steps (Samuelsson, 1983) established that the sulfidopeptide leukotrienes (SRS-A components) and the dihydroxy leukotriene were products of the oxidative metabolism of arachidonic acid by the 5-lipoxygenase pathway. The remarkable proinflammatory potency of these substances when administered to humans (Weiss *et al.*, 1982; Soter *et al.*, 1983) prompted an initial assessment of the effects of dietary enrichment with fish oil-derived fatty acids on the function and products of the 5-lipoxygenase pathway in human leukocytes (Lee *et al.*, 1985a).

There is considerable evidence that EPA and DCHA, which are abundant in fish oil-enriched diets, can substantially modulate the generation and biologic activity of arachidonic acid-derived mediators. A fish oil-enriched diet attenuates the *ex vivo* production of LTB_4 by ionophore-activated human PMN and monocytes and suppresses the chemotactic and endothelial adherence functions of PMN (Lee *et al.*, 1985b; Wong *et al.*, 1986). The suggestion of a reduced incidence of bronchial asthma in Eskimo populations (Herxheimer and Shaefer, 1974), the apparent beneficial effects of EPA supplementation in rheumatoid arthritis (Kramer *et al.*, 1985; Sperling *et al.*, 1986), and the demonstration that a menhaden oil-supplemented diet protects NZB mice from autoimmune nephritis contrast with the findings

that the menhaden diet increases the incidence of experimenal Type II collagen arthritis in the rat (Prickett *et al.*, 1984) and augments or decreases the decrements in pulmonary mechanics in sensitized guinea pigs depending on the route of antigen challenge (Israel *et al.*, 1980; Lee *et al.*, 1985a). The reasons for these contradictory results may be related to the complex interaction between the arachidonic acid and EPA metabolites of the cyclooxygenase and lipoxygenase pathways at the tissue level. Attenuation of a portion of one or both pathways would alter the relative quantities of end-products and could have quite different effects depending upon the pathobiologic mechanisms of the tissue response being assessed.

The beneficial actions of a fish oil-enriched diet on the cardiovascular system seem clear on epidemiological grounds and mechanistically may involve not only attenuation of platelet aggregation and a favorable balance of blood lipids but possibly a reduced participation of leukocytes in chronic and acute inflammatory responses of the vasculature and myocardium, respectively. However, there is no information to securely implicate EPA alone as the beneficial ingredient of a fish oil-enriched diet. Furthermore, there are no detailed dose–response or time-dependent studies for biochemical, functional, and clinical effects as would be appropriate for proceeding to clinical intervention of defined disease states with a fish oil supplement or a homogeneous product. In contrast, the substantial data to indicate that the dietary enrichment with fish oil is relatively nontoxic, except for problems compatible with a somewhat prolonged platelet aggregation time, suggest that further development of this approach to preventive and possible interventive medicine is indicated.

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The Eosinophilic Leukocyte: Structure and Function

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I. Introduction

Although Wharton-Jones in 1846 likely was the first to observe eosinophils in peripheral blood (1), it was Paul Ehrlich who established methods for ready identification of these cells (2). In 1879 Ehrlich stained smears of human peripheral blood cells with aniline dyes and discovered a leukocyte which avidly bound acidic dyes (2). He termed this cell the eosinophil because of the intense avidity of eosin for the leukocyte granules. Later that year Ehrlich found that cells with eosinophilic granules were particularly abundant in the bone marrow of a patient with leukemia and hypothesized that an uncontrolled growth of eosinophils had occurred in the bone marrow. Subsequently, Ehrlich's staining procedure was widely used for examination of peripheral blood, and by the early part of this century the association of peripheral blood eosinophilia with parasitic and allergic diseases had been established. In the past two decades, a rebirth of interest in the eosinophil has occurred, certain functions of the cell have been elucidated, and its roles in health and disease have been partially defined. Here we review information on eosinophil structure and function and provide evidence for the eosinophil as an effector cell for killing helminths and for causing tissue damage in hypersensitivity diseases. We call attention to reviews of this subject elsewhere (3–14).

II. Anatomical Characteristics

The mammalian eosinophil is distinguished by its characteristic granules. Figure 1 shows specific or secondary granules with an electron-dense core and an electron-radiolucent matrix. High power views of the core show a crystalline lattice (15); therefore, the core is likely a single substance. Peroxidase activity is present only in the granule matrix. Two other distinctive types of eosinophil granules have been described. Primary granules are round, uniformly electron dense and characteristically seen in eosinophilic

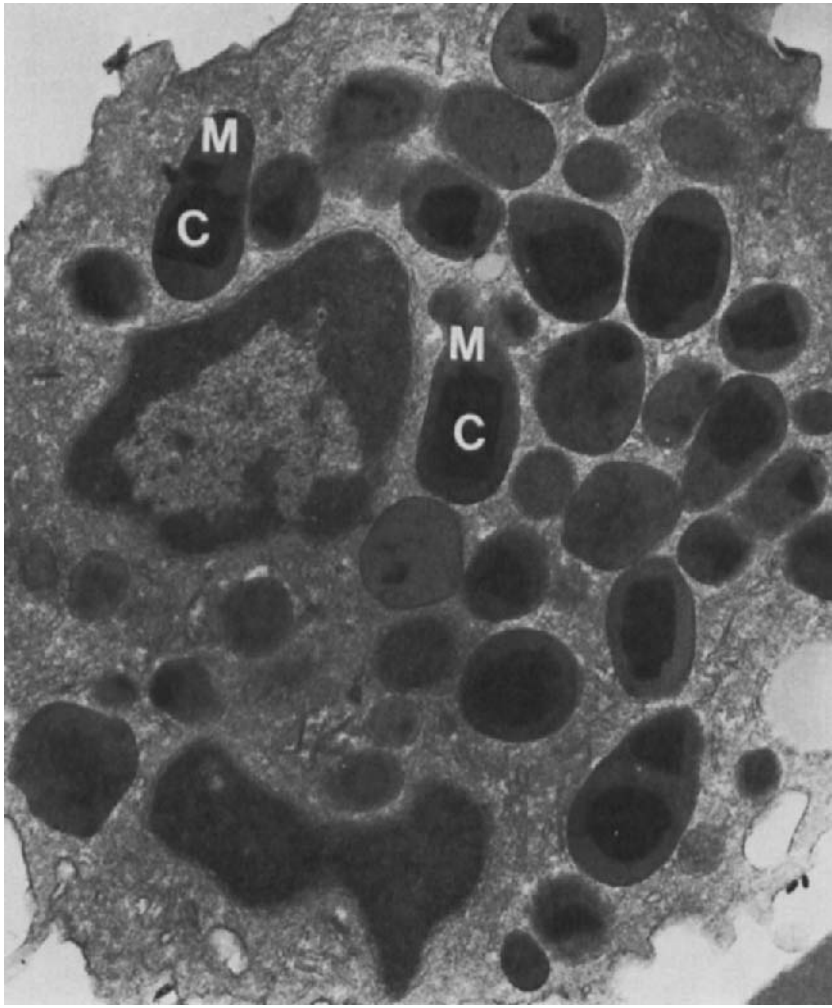

FIG. 1. Electron photomicrograph of a human eosinophil leukocyte with crystalloid-containing granules showing dense cores (C) of various shapes embedded in a less dense matrix (M). ×15,000. (Photomicrograph generously provided by Dr. M. S. Peters.)

promyelocytes (16). Small granules reportedly contain acid phosphatase and arylsulfatase (17).

While granules with crystalloid cores are characteristic of most mammalian eosinophils (the horse eosinophil is a notable exception), cores have not been observed in most lower vertebrates with the exception of certain teleost fishes and some birds (18–20). A primitive reptile, the tuatara,

thought to have remained virtually unchanged for up to 200 million years, possesses eosinophil-like leukocytes which do not contain crystalloids (21). In contrast, the eosinophils of the loach, a fresh-water fish, have crystalline cores at the myelocyte and metamyelocyte stages; the mature eosinophils are characterized by a single, very large granule, 2.5–2.8 μm with a crystalline core (22). Interestingly, the nurse shark, a primitive elasmobranch which has survived 350 million years of evolution, possesses peripheral blood eosinophilic granulocytes containing elongated crystalloids (23). Thus, eosinophilic cells with characteristics similar to those of mammals occur in certain primitive species, suggesting that the eosinophil has contributed importantly to homeostasis over aeons of evolution.

Although eosinophils are usually circular or ovoid when observed by light microscopy, cells with one or more pseudopod-like processes have been observed in human peripheral blood, sputum, bone marrow, and nasal smears (24–26). To emphasize the length and multiplicity of the pseudopods, these cells have been called medusa cells. Because medusa cell formation is enhanced by calcium and magnesium and inhibited by phosphate, citrate, and ethylenediaminetetraacetate, they might not be observed in smears prepared from blood anticoagulated with these agents. Presently, the significance of medusa cells is unknown.

Eosinophil granules usually contain only one or two crystalline cores per granule, but eosinophil granules containing more than five cores per granule have been reported as a familial trait (27). Another inherited, autosomal dominant anomaly of eosinophils is the presence of crystalline inclusions in the cytoplasm (28). The appearance of these inclusions differs from Charcot–Leyden crystals and from the granule's crystalloids. Basophils also show such inclusions, whereas mast cells do not. These findings are interesting because of their apparent familial nature and because they are not associated with overt disease.

III. Eosinophil-Associated Substances

Several of the eosinophil-associated substances discussed below could fit under more than one heading. Therefore, substances have been categorized under the heading by which they are primarily considered at present.

A. Membrane Proteins

1. Immunoglobulin and Complement Receptors

Several studies indicate that eosinophils possess receptors for IgG and C3 fragments (29–36) and the presence of these receptors has been linked to effector functions of the eosinophil toward targets such as parasites (37,38)

and cells (39). Eosinophils from patients with increased numbers of circulating eosinophils possess greater numbers of IgG Fc receptors than eosinophils from normal persons (30,35,39). In contrast, there is no difference in number of complement receptors.

Eosinophil C3b and C4 receptor expression is enhanced by exposure to the eosinophil chemotactic factor of anaphylaxis (ECF-A)[1] tetrapeptides (Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu) and histamine (40,41). In contrast, these same agents do not alter C3b receptors on neutrophils and monocytes, nor are eosinophil IgG Fc receptors affected. [However, others (see 349) have reported an increase of eosinophil IgG Fc receptors after stimulation with ECF-A.] Mixtures of ECF-A and histamine appear to be additive in their effects. Curiously, the number of normal blood eosinophils possessing IgG Fc receptors is only a relatively small proportion of the total, averaging less than 10% (33,39); even in patients with marked eosinophilia the proportion is usually less than 20% (39). Similarly, the proportion of eosinophils with complement receptors ranges from 30% in normal individuals to 40% in patients with parasitic helminth infections (34). Analysis of the eosinophil IgG Fc receptor by surface radiolabeling shows a major protein of 18,000 Da and a minor protein of 16,000 Da (42). In contrast, a human eosinophil IgG Fc receptor has been isolated by affinity chromatography on human IgG-Sepharose columns, and a 43,000 Da macromolecule was found (42a).

Initially, IgM receptors on eosinophils were not detected, but studies using ox erythrocytes coated with rabbit IgM have shown the presence of these receptors (43). Only about 1.5% of freshly isolated eosinophils form rosettes with IgM ox erythrocytes, whereas 27% form rosettes after a 12 hour incubation at 37°C. In contrast, the percentage of eosinophils forming rosettes with IgG ox erythrocytes does not change after incubation. Rosette formation of eosinophils with IgM ox erythrocytes is inhibited by human IgM (and not IgG), and the binding appears to be trypsin sensitive.

Eosinophils also have IgE receptors. Sera from rats infected with *Schistosoma mansoni* mediate killing of schistosomula, and the active factor is destroyed by heating at 56°C for 2 hours, is partially removed by immu-

[1] Abbreviations: AES, anti-eosinophil serum; CLC, Charcot–Leyden crystal; CSF, colony-stimulating factor; EAF, eosinophil activating factor; ECF-A, eosinophil chemotactic factor of anaphylaxis; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; EPO, eosinophil peroxidase; EPX, eosinophil protein X; ESP, eosinophil stimulation promoter; GFA, granulocyte functional antigens; HETE, hydroxyeicosatetraenoic acid; HPR, human pancreatic RNase; LTB_4, 5S-12R-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid; LTC_4, 5S-hydroxy-6*R*-S-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; LTD_4, 5S-hydroxy-6*R*-S-cysteinylglycyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; LTE_4, 5S-hydroxy-6*R*-S-cysteinyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; MBP, eosinophil granule major basic protein; M-ECEF, monocyte-derived eosinophil cytotoxicity enhancing factor; SDS–PAGE, polyacrylamide electrophoresis in the presence of sodium dodecyl sulfate.

nosorption with solid-phase anti-IgE, and is inhibited by soluble aggregated IgE (44). A relatively high percentage, 30–50%, of human and rat eosinophils form rosettes with IgE erythrocytes. Rosette formation is inhibited by aggregated IgE (45) and by antibody to the Fcε receptor (46), but not by aggregated IgG (45) nor by antibody to the C3b receptor (46). Incubation of normal eosinophils with ECF-A increases the proportion of cells forming rosettes with IgE erythrocytes. IgE receptors are not present on human neutrophils.

Sera from *S. mansoni*-infested patients from Kenya [selected because they did not mediate IgG eosinophil killing (37)][2] mediate killing of schistosomula of *S. mansoni* by eosinophils; the active factor is heat labile, and the sera contain IgE antibodies to schistosomula by the radioallergosorbent test. The killing mediated by these sera is inhibited by aggregated IgE and by antiserum to the Fcε receptor. Of interest is the finding that light density eosinophils (vide infra) were more active parasite killers than normodense eosinophils (46). The presence of IgE on human blood and tissue eosinophils has also been shown by flow cytometry (47,48); greater amounts of IgE are found on eosinophils from those individuals with higher levels of serum IgE.

These studies strongly support the existence of an IgE receptor on eosinophils and the ability of IgE to mediate parasite killing by eosinophils. It will be of interest to determine whether IgE-coated eosinophils will degranulate on exposure to allergens; as will be discussed below, evidence exists that toxic eosinophil proteins can damage tissues in certain hypersensitivity diseases. In the rat, eosinophil peroxidase (EPO) release is mediated by IgE antibodies (49); preliminary results suggest that allergens and anti-IgE cause release of EPO from human eosinophils (50).

2. *Receptors for Sheep Erythrocytes*

Eosinophils from patients with parasitic disease form rosettes with sheep erythrocytes (33,51). Curiously, eosinophils from patients with a variety of other diseases including allergic diseases, asthma and rhinitis, hepatitis, and leukemia do not appear to form such rosettes. The significance of this observation is presently obscure; it may serve as a marker for alterations in the eosinophil membrane associated with parasitic infection.

3. *Charcot–Leyden Crystal (CLC) Protein (Lysophospholipase)*

The CLC was initially described in 1853 in a patient with leukemia and later in 1872 in the sputa of patients with asthma. Since then the appearance in tissues and body fluids of these hexagonal bipyramidal crystals has been

[2] It should be mentioned that most sera from *S. mansoni* infected patients in Kenya do mediate killing through IgG antibodies.

regarded as a hallmark of the eosinophil. CLCs have been generated *in vitro* from human eosinophils (52–54); solubilized CLC protein shows a single band after electrophoresis on 1% sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) and a single protein peak by gel filtration in 6 *M* guanidine hydrochloride after reduction and alkylation (53). By these methods the molecular weight of the CLC protein was reported as 12,980. CLC can be solubilized by exposure to nondenaturing solvents; at pH 9 in borate–HCl such solutions contain up to 868 μg/ml (54). PAGE and electrofocusing of CLC protein show multiple bands suggesting that multiple forms are present. These multiple forms of CLC protein are immunochemically identical after analysis by agar gel diffusion and by radioimmunoassay, suggesting that the multiple bands are due to aggregation, carbohydrate heterogeneity, or possibly small variations in primary structure rather than contaminating proteins or markedly different forms of CLC protein (54).

Initial studies of the function of CLC protein were not very revealing; it does not increase vascular permeability, does not contract guinea pig ileum, and does not antagonize the effect of bradykinin or histamine (53). Prior analyses have shown prominent lysophospholipase activity in rat eosinophils (55,56), and subsequent work has shown that purified lysophospholipase forms crystals typical of CLC (57). Lysophospholipase (and thus the CLC protein) appears to be located in the cell membrane (56). For example, centrifugation of disrupted eosinophils on continuous sucrose gradients does not show lysophospholipase activity in granule fractions; rather, activity is found in membrane-containing fractions (along with Mg^{2+}-dependent adenosine triphosphatase activity, a membrane marker) and activity can be sedimented at 100,000 *g*. Lysophospholipase activity of intact cells is inhibitable by cell impermeant probes such as the diazonium salt of sulfanilic acid and *p*-chloromercuribenzene sulfonate. Lastly, during its purification lysophospholipase activity is preserved only if exogenous phospholipids, such as phosphatidylcholine, are present during the assay, a finding consistent with the behavior of other plasma membrane-associated enzymes.

The identity between lysophospholipase from the human eosinophil and CLC protein is supported by the findings that both proteins possess lysophospholipase activity, comigrate on SDS–PAGE, show antigenic identity (58), have essentially the same Michaelis constants of 23.7 and 21.9 μ*M*, respectively, and have virtually identical amino acid compositions with blocked amino-terminal amino acids (59). Thus, lysophospholipase activity is associated with a protein that crystallizes *in vitro* to give CLCs.

Naturally occurring CLCs derived from feces react with antisera to purified lysophospholipase by indirect immunofluorescence; after purification fecal CLCs yield a single protein band that migrates identically to eosinophil lysophospholipase on SDS–PAGE (58). These data strongly support the

hypothesis that CLCs formed *in vitro* are the same as those occurring naturally.

The molecular weight of eosinophil lysophospholipase is reported as 17,000 (59), while that of CLC protein is reported as 13,000 (53). The difference between these molecular weights is likely due to the hydrophobicity of the molecule. The CLC protein molecular weight of 13,000 was obtained using 1% SDS–PAGE, while the lysophospholipase value of 17,000 was obtained with 0.1% SDS–PAGE. Ferguson plots of the electrophoretic migration of reference proteins and eosinophil lysophospholipase in 1% SDS show that lysophospholipase migrates aberrantly rapidly relative to the reference proteins. This aberrant migration is likely due to increased binding of SDS to hydrophobic residues (59).

Historically, CLCs are characteristic of eosinophils, but scattered reports have suggested that they are also associated with basophils. To investigate whether basophils contain CLC protein, they were purified from normal human blood by flow cytometry using antiserum to IgE (60). Three lines of evidence support the conclusion that basophils contain CLC protein: (1) purified basophils form crystals similar to CLC; (2) enrichment for surface IgE-bearing cells also enriched for cells staining positively by immunofluorescence for CLC protein; and (3) basophil CLC protein is immunochemically indistinguishable from eosinophil CLC protein and it is present in basophils in amounts comparable to that in eosinophils (61). These findings indicate that CLC protein can no longer be considered an eosinophil-specific protein. The presence of CLCs in tissues, stool, or sputum might also be derived from basophils.

4. Antigens Recognized by Antiserum to Eosinophils

Another class of membrane proteins associated with eosinophils are the antigens recognized by anti-eosinophil serum (AES). Antisera to mouse (62–64) and guinea pig (65–68) eosinophils have been prepared and used to ablate eosinophils during helminth infections (63,64,66) and during hypersensitivity reactions (67). Specific antisera to human leukocytes, including eosinophils, have also been described (69,70). None of the antigens recognized by these antisera has been characterized.

Recently, murine monoclonal antibodies to human eosinophils have been produced (71,72). Monoclonal antibodies to peripheral blood eosinophils from patients with the hypereosinophilic syndrome recognize determinants on the plasma membranes of blood eosinophils and neutrophils, eosinophil myelocytes, and hemopoietic cell lines (71). Five of the monoclonal antibodies preferentially stain eosinophils of intermediate density, whereas one preferentially stains the light density eosinophils. The percentage of normal eosinophils expressing these antigens was increased by simply incubating

cells *in vitro* or by incubating cells in the presence of monocyte culture supernatants (which activate eosinophils, vide infra). Thus, these antibodies may recognize antigens preferentially expressed on eosinophils during activation and degranulation.

Other monoclonal antibodies may recognize granulocyte functional antigens, or GFA, concerned with cell activation (72). Antigen GFA-2 is expressed on human neutrophils and eosinophils but not on monocytes or lymphocytes and appears relatively late in granulocyte differentiation. The $F(ab')_2$ fragment of monoclonal antibody to GFA-2 increased the killing of schistosomula of *S. mansoni* in a dose-dependent manner. The mechanisms responsible for the stimulation of eosinophils by the $F(ab')_2$ fragment of the monoclonal antibody to GFA-2 are presently obscure.

B. Granule Proteins

Because of the distinctive nature of the secondary or crystalloid-containing granule, it has been assumed that investigation of the properties of its contents could aid in understanding the functions of the eosinophil. Presently, several granule proteins are recognized and their properties and functions will be discussed. As might be predicted from the avidity of the eosinophil granule for the acidic dye, eosin, the granules contain several basic proteins (73). Table I summarizes the characteristics of these proteins.

TABLE I
PROPERTIES OF PRINCIPAL EOSINOPHIL GRANULE PROTEINS

Name	Molecular weight ($\times 10^{-3}$)	*pI*	Activities	Site
MBP	10 (man) 11 (guinea pig)	>11	Toxic to parasites, murine tumor cells, many mammalian cells; causes histamine release from basophils and rat mast cells, neutralizes heparin	Core
ECP	21	>11	Shortens coagulation time, alters fibrinolysis; toxic to parasites; potent neurotoxin; inhibits cultures of peripheral blood lymphocytes; causes histamine release from rat mast cells	Matrix
EDN	17.9	Basic	Potent neurotoxin	Matrix
EPX	18	Basic	Neurotoxin; inhibits cultures of peripheral blood lymphocytes; toxic to parasites	
EPO	71–77 (man) 75 (horse)	>11	In the presence of H_2O_2 + halide kills microorganisms and tumor cells, initiates mast cell secretion, inactivates leukotrienes, causes histamine release from rat mast cells	Matrix

1. Major Basic Protein

a. Biochemical Properties. Solubilization of guinea pig eosinophil granules yields a major basic protein (MBP) so named because (1) it accounts for about 55% of the granule protein, (2) its p*I* is too high to measure; most of the protein applied to a focusing gel migrates into the alkaline wick, and (3) it is proteinaceous in nature (74). MBP has a molecular weight of about 11,000 and shows a marked propensity to polymerize (75). Polymers are multiples of the 11,000 Da monomer, and they depolymerize on exposure to disulfide bond reducing agents. Amino acid analyses of MBP show 13% arginine, 4% tryptophan, 6 half-cystine residues, and 2 reactive sulfhydryl groups. The presence of tryptophan indicates that MBP is not a histone-like protein.

MBPs isolated from human and rat eosinophils (53,76) are rich in arginine and have molecular weights of about 10,000. Immunochemical analyses of these proteins show little cross-reactivity (77). MBP antigenic determinants survive reduction and alkylation in the absence of denaturing solvents; in the presence of 6 *M* guanidinium hydrochloride, reduction and alkylation destroy MBP immunoreactivity (78). Polymerized MBP is only one-tenth as immunoreactive as monomer MBP.

A bovine equivalent of MBP has been described (79); bovine eosinophils were "milked" from the mammary glands of cows infected with *Fasciola hepatica*, and granules were isolated and lysed. A 16,000 Da protein was purified, but amino acid analysis showed no enrichment of arginine. Although this molecule has been referred to as MBP, the paucity of arginine is surprising.

Finally, even though horse eosinophils do not contain crystalloids, an MBP from horse eosinophils has been isolated and has an amino acid composition remarkably similar to that of guinea pig MBP (80). The horse MBP has a molecular weight of 9,000–10,000, a p*I* greater than 10, an arginine content of 10%, and an $E^{1\%}_{1\ cm} = 31.5$ (81). The properties of the various MBP molecules are summarized in Table II.

b. Localization of MBP. i. Eosinophil granule cores. MBP has been localized to the crystalline core of the guinea pig eosinophil granule by immunoelectron microscopy and by isolation of granule crystalloids (82,82a,82b). Immunoelectron microscopy shows that antibodies to MBP bind to the granule core. SDS–PAGE of purified crystalloids shows a band in the expected position for MBP, accounting for 79–91% of the dye staining. Core protein and granule MBP are immunochemically identical as assessed by radioimmunoassay, and all of the core protein can be attributed to MBP. Thus, the core of the eosinophil granule is a crystal of MBP.

Studies of human eosinophils by immunoelectron microscopy show that MBP is present predominantly in granule cores and not within other eosinophil organelles, nor is it in plasma cells, mast cells, lymphocytes, or neu-

TABLE II
COMPARISON OF MBP FROM FIVE SOURCES[a]

	Human	Horse	Guinea pig	Rat	Cow
Molecular weight ($\times 10^{-3}$)	9.2–9.5	9.3–9.9	11.0–11.3	11.0	16.0
p*I*	>10	>10	>10	>10	Basic
$E^{1\%}_{1\,cm}$	26.3	31.5	25.9	—[b]	—
Carbohydrate (%)	<0.8	0	<1	—	0
Arginine (%)	12.0	10.1	13	13	1

[a] Adapted from Barras (81).
[b] Not determined.

trophils (82a). The staining procedure employs tissues fixed in 1% glutaraldehyde and 4% paraformaldehyde and embedded in either Araldite or Spurr; colloidal gold-labeled antibodies are used. Thus, most tissues fixed for electron microscopy can be studied for MBP localization.

ii. Localization of immunoreactive MBP in basophils. The finding of CLC protein in human basophils (61) suggests that MBP might also be present in basophils. Enrichment for surface IgE-positive cells (of which greater than 95% are basophils) by flow cytometry using fluorescein-labeled anti-IgE also enriches for cells staining for MBP by immunofluorescence. These cells are basophils and show MBP staining in a granular pattern. MBP is measurable in extracts of basophils by radioimmunoassay, and the dose–response curves for the basophil extracts are identical to those for eosinophil MBP (83). The MBP content of basophils from normal individuals averages 140 ng MBP/10^6 cells, whereas eosinophils from normal individuals contain 4979 ng/10^6 cells (83). The finding of MBP and CLC protein in basophils points to biochemical similarities between eosinophils and basophils and raises the question of whether CLC protein and MBP might be present in mast cells.

To test whether MBP and CLC protein are present in connective tissue mast cells, purified human lung mast cells (84) and tissue specimens from patients with urticaria pigmentosa have been stained by immunofluorescence for these proteins (85). Neither pulmonary nor cutaneous mast cells stain for CLC protein or MBP, even though eosinophils in the sample preparations show bright staining for both proteins. The inability to identify CLC protein and MBP in mast cells provides further evidence of dissimilarity between basophils and connective tissue mast cells and suggests that this immunochemical difference between them may be used as a means to distinguish these cells (85).

Although the presence of MBP and CLC protein in basophils suggests that these proteins are synthesized by the basophil, an alternative explanation is

that the basophil internalizes MBP and CLC protein. This possibility is suggested by the findings that guinea pig basophils internalize horse EPO and EPO remains associated with granules even when it is removed from the incubation solutions (86). Granule-containing lymphoid cells and mast cells also internalize EPO. While it is possible that the relatively small quantities of MBP identified in human basophils (83) might be derived by internalization, it is more difficult to explain the relatively large amounts of CLC in basophils. For example, the quantities of CLC protein in basophils range from 3 to 32 $\mu g/10^6$ cells while eosinophils contain 9–14 $\mu g/10^6$ cells (61).

The presence of MBP and CLC protein in both basophils and eosinophils is not the only evidence linking these cells. Patients with absence of both eosinophils and basophils (87,88) or with an autosomal dominant anomaly of eosinophils and basophils have been described (28). Eosinophil and basophil levels often rise concordantly in the peripheral blood of parasitized animals (89,90), and these cells appear to cooperate in the rejection of ticks by immune guinea pigs (91). Studies of eosinophil and basophil/mast cell colony formation from human blood show that eosinophil-type colonies, also referred to as Type II colonies (tight accumulations of round, refractile cells), often contain basophils/mast cells (92). Mixed eosinophil–basophil colonies have been found in another study (93). Analysis of Type II colonies showed that they were clonally derived, as judged by linear plating efficiency and by their glucose-6-phosphate dehydrogenase isoenzyme content (94). Type II colonies contain either eosinophils or mixtures of eosinophils and basophils, including cells with mixed basophil–eosinophil granulation. The presence of mixed eosinophil–basophil colonies, clonally derived, suggests the existence of a common precursor. However, this suggestion must be reconciled with the proposal that differentiation of hemopoietic stem cells occurs through a progressive and stochastic restriction in their lineage capabilities (95).

Overall, the above findings support a relationship between basophils and eosinophils. Yet basophils are related to mast cells because they both possess a high affinity IgE receptor and both contain histamine. A recent study of cultured human mast cells showed that they lacked reactivity with monoclonal antibodies specific for granulocyte–monocyte antigens (96). These data have been interpreted as indicating a wide developmental separation between the lineage of mast cells and basophils. Clearly, further studies are needed to clarify the ontogenetic and biochemical relationships of eosinophils, basophils, and mast cells.

iii. Localization of MBP in HL-60 promyelocyte cells. The HL-60 cell line, derived from a patient with acute promyelocytic leukemia (97), has been induced to mature to cells with many of the functional, morphological, and biochemical features of neutrophils or macrophages (98,99). HL-60 cells also differentiate to eosinophils (100–102); for example, HL-60 cells cultured

at pH 7.6–7.8 for 7 days mature to eosinophils (102). Immunofluorescent staining for MBP of unstimulated HL-60 cells shows that 80–85% are positive, many with bright cytoplasmic fluorescence. When HL-60 cells, exposed to dimethyl sulfoxide, differentiate to neutrophils, MBP staining is largely lost. In contrast, cells differentiating to eosinophils retain MBP staining. Immunofluorescent staining of HL-60 cells for CLC protein shows that about 6–21% of cells are positive. CLC protein staining is less intense than that for MBP and, in general, there is not a marked difference between control HL-60 cells and those induced to form eosinophils or to form neutrophils (102). These results suggest that HL-60 cells may be well suited for study of eosinophilopoiesis and that they constitutively produce MBP.

HL-60 cells grown at pH 7.6 for 5–7 days have been induced to form up to 80% mature eosinophils by exposure to 0.5 m*M* butyric acid (103). Butyric acid and its analogs are unique in that all other maturation inducers tested promoted neutrophilic differentiation. These recent findings suggest that lineage commitment and maturation of HL-60 are mechanistically separate processes that can be independently manipulated. Thus, HL-60 cells committed to eosinophil differentiation may be useful to investigate maturational changes in protein synthesis, including gene activation.

iv. Localization of MBP in the placenta. Immunoreactive MBP is elevated in the sera of pregnant women and falls after parturition (104). Examination of placentas for MBP by immunofluorescence shows that MBP is localized to placental X cells and placental giant cells (105). These results will be discussed in greater detail in Section IX.

c. Functions of MBP. i. Toxicity to parasites. Because MBP accounts for over 50% of the eosinophil granule protein, elucidation of its functions should provide insight into the functions of the eosinophil itself. However, early studies of MBP functions were not very revealing (53,75). MBP had only weak antibacterial activity and it did not increase vascular permeability. It precipitated deoxyribonucleic acid, neutralized the anticoagulant activity of heparin, and activated papain, the latter presumably through its two reactive sulfhydryl groups. However, these activities were predictable, considering the basic nature of the molecule. A significant advance has been the discovery that MBP as well as certain other basic proteins, including protamine, have damaged schistosomula of *S. mansoni* (106). MBP has bound to the membrane of the schistosomula and caused their disruption (Fig. 2). Heparin has inhibited the uptake of MBP onto the schistosomula and the subsequent damage. When eosinophils in the presence of antibody have attacked the schistosomula, they have deposited MBP on the surface of the parasites, and MBP could be detected in culture supernatants. Heparin at high concentrations partially blocks eosinophil-mediated damage to the schistosomula (A. E. Butterworth, personal communication). The latter find-

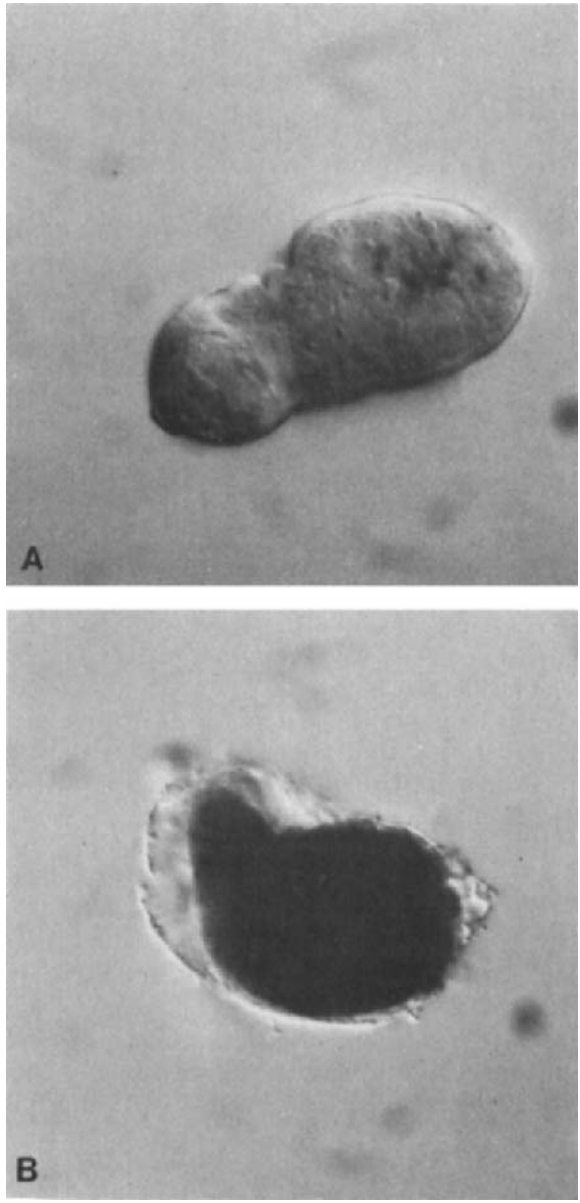

FIG. 2. Photomicrographs of schistosomula of *S. mansoni* showing effect of incubation with MBP. (A) Shows a control schistosomulum that was motile and translucent. (B) Shows a schistosomulum treated with MBP; note the detached and ballooned membrane and the staining with toluidine blue. ×230. (Photomicrographs generously provided by Dr. A. E. Butterworth.)

ing is in keeping with prior observations by electron microscopy that the eosinophil granule contents are liberated into a confined, relatively inaccessible space between the cell and the parasite (107).

Killing of schistosomula occurs as a two-stage reaction (108,109). The initial adherence of the eosinophil is temperature independent and involves the cell's Fc receptors. The eosinophil, but not the neutrophil, then undergoes a further temperature-dependent step rendering binding to the worm irreversible. The strong stable binding of the eosinophil to the parasite is associated with degranulation and release of granule proteins (109), and it is hypothesized that the stable binding is mediated by sticky cationic proteins. Support for this concept has been provided by the following findings: (1) MBP or protamine enhances adherence of eosinophils and neutrophils to schistosomula, (2) adherence of eosinophils to schistosomula by concanavalin A is reversed by α-methylmannoside, is not associated with damage to the parasites, and does not lead to eosinophil degranulation, but (3) if the calcium ionophore A23187 is added to concanavalin A-bound eosinophils, adherence is no longer reversible, the schistosomula are damaged, and MBP is released into the medium.

Studies of the effect of MBP on newborn larvae of *Trichinella spiralis* have shown that all larvae cultured with MBP at concentrations of 5×10^{-5} *M* or higher are rendered immobile (110). The effect of MBP is detected as early as 2 hours after treatment when the larvae become progressively stiffened and sluggish. Other basic proteins did not cause damage except for polyarginine, which killed 20% of larvae in 24 hours. In contrast, muscle stage larvae are quite resistant to MBP, surviving for hours without loss of motility (Gleich and Loegering, unpublished).

The bovine MBP-like molecule is toxic to juvenile forms of *F. hepatica* at concentrations as low as 1×10^{-6} *M* (79). Protamine sulfate and polyornithine also are toxic to *F. hepatica* although less so than the bovine MBP.

MBP also kills a protozoan parasite, the bloodstream trypomastigote stage of *Trypanosoma cruzi* (111). Other basic proteins, such as protamine and poly-L-arginine, are also active, but MBP is 5-fold to 10-fold more active. Heparin inhibits MBP toxicity in a dose-related manner, and antibody to MBP inhibits toxicity up to 82%. Surprisingly, heparin also inhibits killing of trypomastigotes of *T. cruzi* by eosinophils and 125–312 units/ml causes up to 90% inhibition (112). Amastigote forms of *T. cruzi*, the intracellular form of the organism, are ingested by eosinophils and damaged (113); MBP is deposited on the ingested amastigotes. Heparin, dextran sulfate, and chondroitin sulfate inhibit intracellular killing of amastigotes by eosinophils. MBP itself kills amastigotes and its toxicity is inhibited by specific antibody.

ii. Toxicity to mammalian cells. MBP is toxic to murine tumor cells (106) and to many mammalian cells in a dose-related manner (114). Concentra-

tions of MBP in this toxic range are present in body fluids from patients with diseases associated with eosinophilia; this finding suggests that the eosinophil releases granule proteins and causes tissue damage in hypersensitivity diseases. Tests of the hypothesis that the eosinophil damages cells and impairs organ function in disease states will be summarized in Section VIII.

iii. Stimulation of histamine release from basophils and mast cells. Incubation of peripheral leukocytes with native MBP, the form of the molecule with reactive sulfhydryl groups, causes dose-dependent histamine release from peripheral blood basophils in a concentration range from 5×10^{-7} to 7.5×10^{-6} *M* (115). Analysis of histamine release kinetics shows that histamine increases for approximately 40 minutes; half-maximal secretion occurs at about 17 minutes. Native MBP is 10-fold more active than reduced and alkylated MBP; reduction and alkylation are carried out to stabilize monomer MBP by preventing polymerization. The release process is Ca^{2+}, temperature and energy dependent, and thus not cytotoxic. Experiments showing that histamine release occurs from purified blood basophils establishes the basophil as the target of the MBP effect. MBP is also active on rat peritoneal mast cells, but here histamine release is essentially complete after 1 minute (115). While individual donors differ in the sensitivity of their basophils to MBP activation, cells from patients with atopic diseases and normal persons do not differ (116).

Other experiments have found that MBP, but not EPO, EDN, or ECP, stimulates histamine release from human mononuclear cells (117). In contrast, MBP, ECP, and EPO but not EDN stimulate histamine release from rat mast cells. A recent report confirmed the ability of MBP to cause histamine release from peripheral human blood cells, but also reported that ECP was active (118).

iv. Effect on coagulation. MBP neutralizes the effect of heparin on blood clotting as does protamine; and MBP increases the clotting time from 3- to 5-fold (75). The target of MBP action on coagulation is not known.

2. *Eosinophil Cationic Protein*

Analyses of human leukemic myeloid cells have shown seven cationic components (119). In these experiments leukocytes from patients with myeloid leukemia were disrupted, the granule fraction obtained by centrifugation, and the granules extracted at pH 4. Extracts were purified by gel filtration, electrophoresis, and ion-exchange chromatography. Four components had molecular weights of 25,500–28,500 and similar amino acid compositions. The remaining three had molecular weights of 21,000–29,000 and showed immunologic identity. The amino acid composition of component 5 differed from those of components 1, 2, and 3. Subsequent investigations

have shown that components 5, 6, and 7 are derived from eosinophils (120). The major constituent (component 5) has a molecular weight of 21,000 and has been termed the eosinophil cationic protein (ECP) (121).

a. Biochemical Properties. ECP has the following properties: it has an isoelectric point greater than pH 11, it contains 11% arginine and 10 half-cystines, SDS–PAGE shows a single polypeptide chain (121), and it contains 2.5 mol of zinc per mol protein. By several criteria, ECP and MBP have different properties and are distinct molecules. Their amino acid compositions are quite different, the smaller MBP molecule contains more lysyl, glycyl, and tryptophanyl residues than ECP and no methionyl residues (53,121). Direct comparison of MBP and ECP shows that they elute at different volumes from Sephadex G-50 (Fig. 3) and migrate as distinct bands on SDS–PAGE with differing molecular weights (122). Immunochemical analyses do not demonstrate any appreciable cross-reactivity. Comparison of the quantities of MBP and ECP from granules shows between 6 and 64 times more MBP on a weight basis (122). Thus, MBP and ECP are distinct molecules with different properties and are present in eosinophil granules in differing amounts.

Purification of ECP by high-performance liquid chromatography reveals five separate components each reactive with antibody to ECP. These components show almost identical amino acid compositions and have molecular weights of 16,700 and 19,500 (123). Analysis of the amino terminus of one of the components shows the following sequence:

```
          10        20        30
          ↓         ↓         ↓
RPXQFTRAQWFAIQHISLNPRRCTIAMRAINNY
```

A computer search has shown that this 33 residue sequence is unique and no homologies have been detected. In the same study labeling of ECP was accomplished by *in vitro* incubation of bone marrow cells with [^{14}C]leucine followed by immunoprecipitation with specific antibody. Biosynthesis of a 22,000 Da ECP molecule was demonstrated and this was processed into an 18,000 Da species (123).

In another study, ECP was purified from eosinophil granules and two peaks of antigenic activity were eluted from heparin-Sepharose (Fig. 4) (124). These two species of ECP, termed ECP-1 and ECP-2, show striking heterogeneity on SDS–PAGE; ECP-1 shows a major band at 18,300 Da and a minor band at 21,400 Da, and ECP-2 shows three bands, a doublet at 16,000 Da and another band at 16,900 Da. Digestion of ECP-1 with *endo*-β-*N*-acetylglucosaminidase F (125) reduces the molecular mass to 16,000; digestion of ECP-2 removes the 16,900 Da form. This result suggests that the electrophoretic heterogeneity of ECP may be, in part, a consequence of

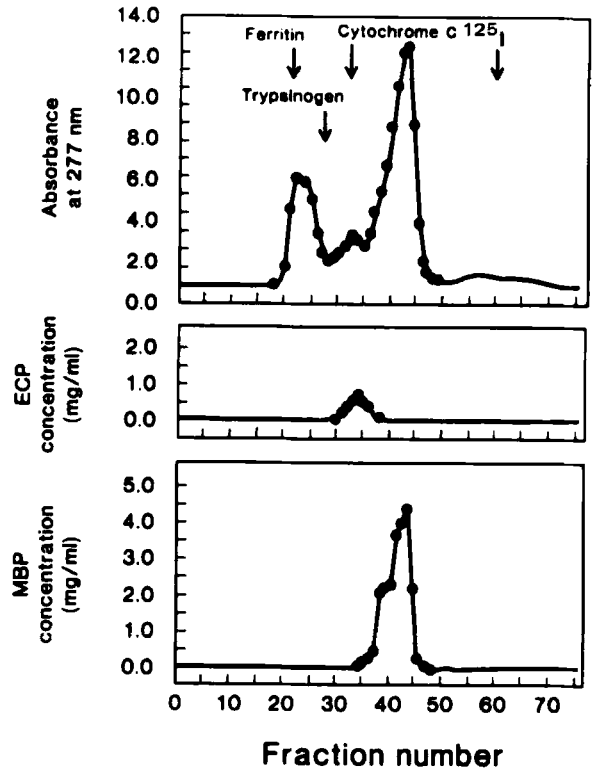

FIG. 3. Analysis of MBP and ECP levels in Sephadex G-50 column fractions of acid-solubilized eosinophil granules. Individual column fractions were analyzed for MBP and ECP by radioimmunoassay. Molecular weight markers for this column included ferritin (750,000), trypsinogen (24,000), cytochrome *c* (12,400), and ^{125}I. The upper panel shows the protein absorbance profile at 277 nm, the middle panel shows the quantity of ECP in the individual column fractions, and the lower panel shows the quantity of MBP in the individual column fractions. [Used with permission from Ackerman *et al.* (122).]

carbohydrate additions. Two-dimensional nonequilibrium pH gradient electrophoresis and SDS–PAGE show charge heterogeneity, suggesting the addition of sialic acid or other unidentified posttranslational modifications.

Amino-terminal sequence through residue 59 (Fig. 5) revealed identical sequences for ECP-1 and ECP-2 (124). As discussed in Section III,B,3, the ECP sequence has quite marked homology with EDN and most surprisingly with ribonuclease. Figure 6 shows that the homology with ribonuclease extends from residues 3 to 65. Each of the four cysteines aligns perfectly and when so aligned, it is apparent that the proteins have not diverged from each other in a completely random manner. In contrast to the amino terminal

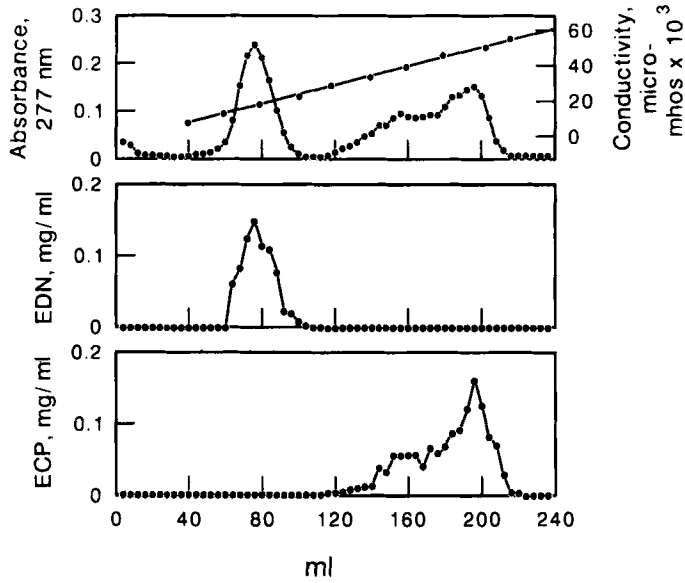

FIG. 4. Purification of EDN and ECP on heparin-Sepharose. Fractions from the second peak of solubilized eosinophil granules from Sephadex G-50 fractionation (see Fig. 3) were pooled, dialyzed against 5 m*M* Na_2HPO_4–NaH_2PO_4/75 m*M* NaCl, pH 7.4, and applied to heparin-Sepharose. A linear NaCl gradient was applied with a 1.5 *M* NaCl limit. EDN and ECP were analyzed in fractions by radioimmunoassay. [Used with permission from Gleich *et al.* (124).]

```
                          10                  20
EDN:  K P P Q F T X A Q W F E T Q H I X M T S
      : P P Q F T : A Q W F : : Q H I : : : :
ECP:  R P P Q F T R A Q W F A I Q H I S L N P

                          30                  40
EDN:  Q Q C T N A M Q V I N N Y Q R R C K N Q
      : : C T  A M : : I N N Y :  R C K N Q
ECP:  P R C T I A M R A I N N Y R W R C K N Q

                          50                  60
EDN:  N T F L L T X F A N V V N T C X X X X X C
      N T F L  T : F A N V V N : C : : : : X X C
ECP:  N T F L R T T F A N V V N V C G N Q S X X C
```

FIG. 5. NH_2-terminal amino acid sequences for EDN and ECP. The homology between the proteins is shown by the middle line; positions where the sequences are identical are shown by the amino acid itself and positions where a conservative replacement exists are shown by a colon. The perfect correspondence between the cysteines of EDN and ECP has been emphasized by shading. The *Z* score for similarity was 35.7; *Z* scores >10 are considered significant. X, No amino acid identified. [Used with permission from Gleich *et al.* (124).]

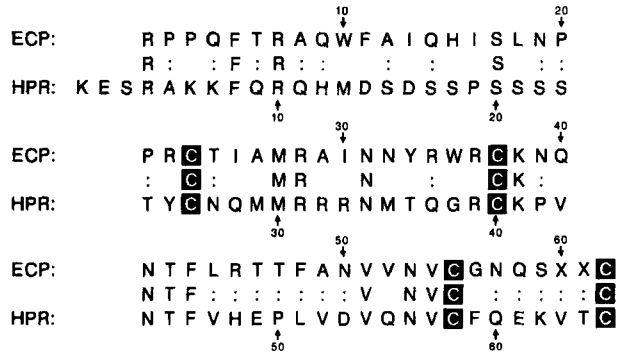

FIG. 6. Sequence homology between ECP and human pancreatic RNase (HPR). The middle line compares these sequences, as in Fig. 5. The Z score for similarity was 11.5. The sequences have been aligned for optimal homology. The perfect correspondence between the cysteines of RNase and EDN has been emphasized by shading. [Used with permission from Gleich *et al.* (124).]

region, the 19 amino acids between the second and third cysteines (ribonuclease residues 40–58) are highly conserved. This region contains the ribonuclease binding site (126,127). Similarly, the lysine at position 38 of EDN and ECP matches the lysine at position 41 of ribonuclease, a catalytically active residue. Although the sequence analyses of EDN and ECP are imcomplete, the marked homology between these proteins and ribonuclease, in regions of the molecule that are important for ribonuclease function, suggests that EDN and ECP may have ribonuclease-like enzymatic activity.

Monoclonal antibodies to ECP show differences between stored and secreted forms of the molecule (128). Antibody EG1 recognizes both the stored and secreted forms of ECP, whereas EG2 recognizes only secreted (or extracted) ECP. Antibody EG2 does not react with resting eosinophils; however, EG2 reacts with eosinophils after phagocytosis and after activation with eosinophil cytotoxicity enhancing factor (vide infra). Also, EG2 reacts with eosinophils from patients with the hypereosinophilic syndrome. Thus, EG2 appears to recognize activated eosinophils, and it has been used to identify these cells from tissue and blood in disease (128–130).

b. Localization. Because MBP forms the eosinophil granule crystalloid, one would predict that ECP would be present in the matrix. By use of a colloidal gold immunoelectron microscopic assay, ECP has been localized to the granule matrix (82a,82b). This localization has been performed in the same experiments in which MBP has been localized to the granule core.

c. Functions. i. Effects on coagulation and fibrinolysis. ECP does not

possess bactericidal or esterolytic activity; it does not contract the guinea pig ileum, nor does it have any inhibitory effect on histamine-induced or guinea pig anaphylotoxin-induced contractions of the ileal muscle (120). As expected for such a basic molecule, ECP binds to heparin and neutralizes its anticoagulant activity (121). ECP shortens the coagulation time of plasma in a dose-dependent manner (131). This stimulatory effect of ECP is not seen in plasma deficient in Factor XII; it is seen in plasma deficient in Factors V, VII, VIII, IX, X, and XI, suggesting that Factor XII is the target of ECP interaction. At high concentrations, ECP prolongs the clotting time. The activity of kallikrein is also enhanced by ECP.

ECP alters fibrinolysis as shown by enhancement of urokinase-induced plasminogen activation (132). In contrast, plasminogen activation by streptokinase is abolished by ECP; this effect evidently is due to the formation of a precipitate between streptokinase and ECP. The enhancement of plasminogen activation is not associated with the formation of a complex between ECP and plasminogen.

ii. Parasite killing. ECP is a potent toxin for schistosomula of *S. mansoni* (133) and in a comparative test ECP was 8- to 10-fold more active than MBP (134). The ECP killing of schistosomula is qualitatively different than that of MBP. ECP produces complete fragmentation and disruption of schistosomula, whereas MBP produces a distinctive ballooning and detachment of the tegumental membrane (Fig. 2). The toxicity of MBP is destroyed by heating at 56°C for 4 hours; ECP activity is not affected. To determine the relative contribution of MBP and ECP, fractions of acid-soluble eosinophil granule preparations from Sephadex G-50 have been tested for toxicity. Schistosomula are killed by fractions containing MBP, but not by other fractions containing ECP; the ECP fractions contain ECP at concentrations below the toxic level. Thus, even though on a molar basis ECP is a more potent helminthotoxin for schistosomula than MBP, MBP by virtue of its abundance in the granule accounts for the bulk of the toxicity in Sephadex G-50 fractions of acid-solubilized granules from patients with marked eosinophilia (134). Clearly, experiments are needed to test whether these molecules act synergistically, because during eosinophil degranulation they would be applied to the target simultaneously and congruently.

A test of ECP on lung stage schistosomula showed that it caused paralysis, but not death at high concentrations; the effect was reversible and the worm did not show gross pathological abnormalities (135).

iii. Neurotoxicity. As discussed below in more detail, extracts of eosinophils produce a neurotoxic reaction when injected into the cerebrospinal fluid or the brain of rabbits or guinea pigs (136). The guinea pig is exquisitely sensitive to this neurotoxic activity and ECP is a potent neurotoxin (137).

iv. Effect on lymphocyte proliferation. Culture of human peripheral

blood lymphocytes in the presence of ECP shows a dose-dependent inhibition of proliferation induced by phytohemagglutinin and by a one-way mixed lymphocyte reaction (138). The effect is reversible and may involve suppressor cells. At 10^{-7} *M*, the peak concentration of ECP tested, a 33% reduction from control in the phytohemagglutinin response and a 50% reduction from control in the mixed leukocyte culture were found. Preincubation experiments showed that the effect in the mixed leukocyte reaction was on the responder cell. Also, an enhancement of suppressor cell activity was found. The results suggest that ECP has an effect on lymphocyte proliferation and that eosinophils might exert regulatory effects on lymphocytes. The eosinophil granule protein termed EPX (vide infra) was also tested in these experiments and had effects comparable to ECP (139); EPX will be discussed in Section III,B,3.

v. Mechanisms of toxicity. Analysis of the mechanism of ECP action on lipid vesicles shows that it may damage cells by a colloid-osmotic process (140).

3. *The Eosinophil-Derived Neurotoxin (EDN)*

Eosinophils contain a powerful neurotoxin that can severely damage myelinated neurons in experimental animals (141–143). M. H. Gordon first described this neurotoxic reaction in 1932 (141), and it is presently referred to as the "Gordon phenomenon." Patients with the idiopathic hypereosinophilic syndrome and cerebrospinal fluid eosinophilia exhibit varied neurologic abnormalities (144–149), and the possibility exists that neurotoxic eosinophil granule proteins are important in central nervous system disease in man.

a. Biochemical Properties. EDN has been partially purified by centrifugation of sonicated human eosinophils and by gel filtration of the supernatant (136). Fractions with neurotoxic activity elute at approximately 15,000 Da. Neurotoxic activity withstands lyophilization and dialysis, but is destroyed by heating at 90°C. Subsequently, extracts of whole human eosinophils and purified eosinophil granules have been fractionated by gel filtration at acid and alkaline pH (150). Fractions have been analyzed by SDS–PAGE and by their ability to produce the Gordon phenomenon by intrathecal injection into rabbits. Fractionation of granule extracts on Sephadex G-50 at pH 4.3 yields three major peaks (Fig. 3). Eosinophil enzymes including peroxidase are in peak 1 while MBP is in peak 3. Neither peak 1 nor peak 3 protein produces the Gordon phenomenon. Peak 2 fractions, rechromatographed on Sephadex G-50 at pH 7.4, show a single peak and injection of this substance causes the Gordon phenomenon. The active material gives a single band on SDS–PAGE with a molecular weight of about 18,000.

EDN has been further purified on heparin-Sepharose (Fig. 4) and the amino-terminal sequence determined (Fig. 5) (124). EDN and ECP show

very marked sequence homology and 37 of the 54 amino-terminal residues are identical. Nonetheless, there are major differences in the partial sequences which likely explain the difference in reactivity with polyclonal (122) and monoclonal antibodies (124). As discussed above (see ECP) both EDN and ECP are homologous to ribonuclease. Thus, EDN and ECP are related proteins, and their homology with ribonuclease suggests that they are derived from genes associated with the ribonuclease family.

A potentially new protein, called eosinophil protein X (EPX), has been described (139) and may be the same as EDN. EPX produces the Gordon phenomenon (137) and it has a molecular weight similar to EDN. The amino acid composition of EPX shows a high content of arginine (139). EPX and ECP have similar effects on lymphocytes (138). Interestingly, EPX is highly toxic to lung stage schistosomula of *S. mansoni* and damages the subtegumental musculature and internal tissues of the parasite, rather than the surface syncytium and tegumental outer membrane (135).

b. Localization. Extracts of highly purified eosinophil granules produce the Gordon phenomenon (150); this result points to the eosinophil granule as the source of EDN. Because MBP forms the crystalloid, it seems likely that EDN is in the granule matrix and immunoelectron microscopy has confirmed the presence of EDN in the granule matrix (82a).

c. Functions. The only known function of EDN is its ability to provoke the Gordon phenomenon. When injected intrathecally into rabbits or guinea pigs, EDN produces a predictable syndrome which begins with stiffness, most noticeable in the forelimbs, mild ataxia, followed by incoordination and marked ataxia so that animals have difficulty remaining upright. The final phase of the Gordon phenomenon is associated with severe weakness and muscle wasting (136). Some animals develop nystagmus and jerky, repetitive head movements. No evidence of abnormalities of higher level functions is observed; animals remain alert and eat and drink in a normal fashion, provided food and water are placed within reach. The latent period between injection of EDN and the onset of neurologic manifestations ranges from 3 to 11 days (136,150).

The histopathology of the Gordon phenomenon in rabbits has been thoroughly described (136,150). On light microscopy, the histologic abnormalities are concentrated in the cerebellum, pons, and spinal cord. A hallmark of the Gordon phenomenon is the disappearance of cerebellar Purkinje cells. In addition, the white matter of the cerebellum, pons, and spinal cord shows a marked spongiform change; the grey matter remains essentially normal. In the guinea pig the abnormalities are similar (137) and the Purkinje cell is not the only cell affected. A clear-cut reduction in numbers of cells in the molecular layer is also apparent, possibly involving basket cells, stellate cells, and glia; the Golgi II cells are absent in the underlying granule layer.

In a study of the neurotoxicity of ECP and EPX in guinea pigs, ECP was 100-fold more potent that EPX (137). A test of the comparative neurotoxicity of ECP and EDN in rabbits showed that they had essentially indistinguishable toxicity (124). If EDN is EPX, then the difference in these results would be attributable to a species difference between guinea pigs and rabbits. CLC protein (lysophospholipase), EPO, and MBP do not produce the Gordon phenomenon (124,150).

When EDN has been tested for toxicity to schistosomula of *S. mansoni*, little reactivity has been found (134). In contrast (as noted above) EPX, which may be the same as EDN, is highly toxic to lung stage schistosomula of *S. mansoni* (135).

C. Eosinophil-Associated Enzymes

1. Eosinophil Peroxidase (EPO)

EPO is localized in the granule matrix of numerous species, including laboratory animals (151–153) and humans (82b,154). The intensity of peroxidase staining is so great that it can be used to enumerate eosinophils, as in the automated continuous-flow cytometers used for differential counts (155,156). Interestingly, the eosinophils of many members of the cat family including the lion, the tiger, and the domestic cat do not contain peroxidase (157,158).

a. Biochemical Properties. EPO, partially purified from rat eosinophils, shows spectral differences from neutrophil myeloperoxidase and a Soret maximum of 403 nm for oxidized EPO and 437 nm for reduced EPO (159). EPO purified from marrow cells of guinea pigs by detergent extraction, ion-exchange chromatography, and gel filtration (160) is homogeneous by immunodiffusion and exists as a monomer of 75,000 Da and a dimer of 150,000 Da. Both monomer and dimer have the same specific activity, the same absorption spectrum, a Soret maximum at 425 nm, and the same absorbance ratio at 415 nm and 280 nm. In the guinea pig EPO evidently consists of a single polypeptide chain as indicated by a single band in SDS–PAGE. Horse EPO has been purified both from intact eosinophils (161,162) and from their granules (162); it has a Soret maximum of 417 nm with a shift to 433 nm after reduction. The 415/280 nm ratio is 1.05 and the isolated protein is strongly basic; it migrates to the alkaline wick on isoelectric focusing.

Human EPO has been purified by several groups (163–166) employing as the starting material either whole blood leukocytes (163–165) or eosinophil granules (166). EPO has a molecular weight of 71,000 by sedimentation equilibrium (165) and 77,000 by gel filtration (166), consists of two subunits (58,000 and 14,000 Da) in a 1:1 stoichiometry, and has a Soret band at 412 nm (165). Two other reports show similar findings with molecular weights of 50,000 (164) and 52,000 (166) for the heavy chain and 15,500 (164) and 15,000

(166) for the light chain and a Soret band for the native enzyme at 415 nm. Amino acid composition shows a high content of arginine, leucine, and aspartic acid (165,166). The isoelectric point of EPO is greater than 11 (166). A specific antiserum to EPO has been prepared which does not cross-react with ECP, EPX, myeloperoxidase [in keeping with a prior report (167)], elastase, lactoferrin, lysozyme, and chymotrypsin-like cationic protein (166). By radioimmunoassay, the cell content of EPO is 15 $\mu g/10^6$ eosinophils (166).

The biosynthesis of human EPO has been studied in marrow cells from patients with eosinophilia by internal labeling with [^{14}C]leucine (168). Labeling of 53,000 and 25,000 Da subunits was demonstrated. The 25,000 Da subunit may represent a precursor of the 15,000 Da light chain. Labeling has also been detected in two polypeptides of 78,000 and 72,000 Da and these forms of EPO may represent precursor polypeptides.

Studies of the EPO heme prosthetic group have revealed a molar extinction coefficient, $E_{413} = 110\ mM^{-1}\ cm^{-1}$ (165). Electron paramagnetic resonance spectra has shown high-spin ferric heme signals with rhombic symmetry for the heme group and a content of heme iron of 13.2×10^{-17} mol/eosinophil (169). The heme–ligand structure of eosinophil peroxidase is closely related to lactoperoxidase, whereas myeloperoxidase resembles catalase (165). Analysis of horse and human EPO by resonance-enhanced Raman spectroscopy leads to the conclusion that the heme prosthetic group is a high spin, six coordinate protoporphyrin (170). The Raman spectrum shows a clear difference between EPO and lactoperoxidase and points to a stronger axial sixth ligand in EPO.

b. Localization. As noted above, EPO is localized to the matrix of crystalloid-containing eosinophil granules (151–154).

c. Deficiency. EPO is not present in eosinophil granules of the cat family (157,158) and in eosinophils of certain human kindreds (171–173). The first cases of EPO deficiency were identified in Yemenite Jews and suggested an autosomal recessive mode of inheritance (172,173). EPO deficiency is accompanied by nuclear hypersegmentation, by reduction in the degree of granulation, and by apparent loss of phospholipids as judged by Sudan black B staining (172). Myeloperoxidase deficiency is also associated with a failure of neutrophil granule staining with Sudan black B; in contrast, eosinophils do show staining with Sudan black B in myeloperoxidase deficiency (172a). EPO deficiency has not been associated with any distinctive clinical symptoms, and it has been proposed as marker in population genetics (174). A study of 63,465 persons in the Rehovot district of Israel showed the highest gene frequency among Yemenite Jews followed in decreasing order by Jews of North African and Iraqui–Persian extraction and by the Arab population. The gene is rare in Askenazi Jews (174). Ultrastructural examination of EPO-

deficient human eosinophils shows prominent cores and a relatively small matrix zone (175).

Partial EPO deficiency has also been described (174) and detailed cytochemical studies have shown deficiency of peroxidase in both eosinophils and neutrophils in a patient with ceroid lipofuscinosis (Kuf's disease) (176).

Often the study of peroxidase deficiency requires that a distinction be made between EPO and myeloperoxidase, but one assay commonly used for myeloperoxidase measurement, namely guaiacol peroxidation, is profoundly affected by small numbers of blood eosinophils. Inclusion of 3-amino-1,2,4-triazole in the guaiacol assay inhibits EPO but not myeloperoxidase activity and permits use of the guaiacol assay in the detection of myeloperoxidase deficiency, especially partial deficiency (177). Contrariwise, EPO can be measured independently of myeloperoxidase in mixed granulocyte preparations (178).

d. Function. i. Direct toxic effects of the EPO + H_2O_2 + halide system. Myeloperoxidase catalyzes the oxidation of many substances by hydrogen peroxide. Earlier work has established that myeloperoxidase plus hydrogen peroxide and iodide iodinate kill bacteria, and that the activity of this system is increased in phagocytizing neutrophils (179). The combination of myeloperoxidase, H_2O_2, and halide also kills viruses (180), mycoplasma (181), and fungi (182).

Because eosinophils generate H_2O_2 (183–185) and have an active iodination capability (186), the killing of microorganisms by the EPO + H_2O_2 + halide system has been tested. An early study showed that eosinophil extracts kill *Staphylococcus aureus* and *Escherichia coli,* but only in the presence of iodide, not chloride (187). Purified EPO kills *E. coli* in the presence of H_2O_2 and chloride as well as iodide and bromide (188). The difference between these results may be the presence of gelatin in the reaction mixture; the latter study showed that gelatin and albumin inhibit killing of *E. coli* by the EPO system. In general, EPO is not as effective with chloride as it is with iodide, and of the halide group iodide > chloride > fluoride (S. J. Klebanoff, personal communication). Subsequent work has shown that EPO in the presence of H_2O_2 and halide kills schistosomula (189), toxoplasma (190), trypanosoma (191), mycobacteria (192), mast cells (193), and tumor cells (194). These studies indicate that this system can mediate toxicity to numerous targets.

ii. EPO effects on mast cells and their granules. EPO binds to mast cell granules, and the EPO–mast cell granule complex catalyzes iodination of proteins and killing of microorganisms (195). The possibility exists that such complexes form extracellularly and affect the inflammatory response.

EPO supplemented by H_2O_2 and halide induces mast cell degranulation and histamine release (193). This reaction is noncytotoxic at low EPO con-

centrations, but at high concentrations ultrastructural evidence of mast cell damage is evident. The EPO–mast cell granule complex is more effective than free EPO itself in the stimulation of mast cell secretion. These findings lead to the proposal that eosinophils, either by secretion or by cell lysis, release EPO and that EPO in the presence of H_2O_2 (generated by eosinophils or other phagocytes in the area), chloride, and iodide initiates mast cell secretion (193). At pH 7.4, EPO is active at physiological concentrations of chloride plus 10^{-6} *M* iodide; this iodide concentration is above that present in extracellular fluid. Examination of rat mast cell degranulation by EPO + H_2O_2 + halide has shown that granules become swollen and some open to the outside of the cell (196). Other granules have shown reduced electron density and pores have formed in the surface membrane. At high EPO concentrations a frank cytotoxic disruption of the mast cell has occurred. These results suggest a role for the EPO + H_2O_2 + halide system in the inflammatory response. Whether the proposed reactions occur in disease requires further study. One report has shown leakage of EPO from eosinophil granules into the cytoplasm and apparent extracellular release following allergen provocation of human nasal membranes (197).

iii. Effects of EPO bound to effector cells. Further studies of the EPO + H_2O_2 + halide system have indicated that binding of EPO to microbes such as *S. aureus* (198), *Toxoplasma gondii* (190), and *T. cruzi* (191) markedly potentiates their killing by mononuclear phagocytes. Interestingly, tumor cells adsorb EPO and this binding potentiates their lysis by H_2O_2 (199). The EPO-coated tumor cells are spontaneously lysed by macrophages and this damage is inhibited by catalase (indicating a need for H_2O_2) and by azide (indicating a need for enzymatically active EPO). These results point to a synergistic action between the cytophilic cationic EPO and H_2O_2, spontaneously released from macrophages, in destroying tumor cells (199).

These findings, plus the observations that MBP is toxic to tumor cells (106), suggest that the eosinophil may function to limit the spread of tumors. Infiltration of tumors by eosinophils including lung, skin, intestine, and breast has been reported since the last century, and several studies have claimed that the mortality associated with carcinomas decreases as the degree of eosinophil infiltration increases (200–202). This possibility requires further study especially in view of observations that eosinophils may lose their morphologic integrity in tissues (203); in other words simply counting the number of stainable eosinophils may not be sufficient to judge the participation of the cell in disease.

iv. Effect on leukotrienes. Incubation of leukotriene C_4 (LTC_4) or leukotriene D_4 (LTD_4) with EPO + H_2O_2 + halide rapidly decreases bioactivity on smooth muscle (204). Similarly, EPO + H_2O_2 + halide decreases the chemotactic activity of leukotriene B_4 (LTB_4). Inactivation or deletion of any of the components of the EPO + H_2O_2 + halide system leads to a decrease

in the degradation of LT activity. Although the mechanism of inactivation is obscure, LTC_4 and LTD_4 each contains thioether linkages connecting cysteine with carbon-6; the thioether group of methionine is sensitive to oxidation by myeloperoxidase + H_2O_2 + halide system with formation of a sulfoxide derivative (205).

Another study of the effect of EPO + H_2O_2 + halide on LTC_4 has shown that LTC_4 is converted to two isomers of LTB_4, 5(*S*),12(*R*)-"all *trans*"-LTB_4 and 5(*S*),12(*S*)-"all *trans*"-LTB_4 (206). These LTB_4 isomers possess less than 1/100 of the chemotactic activity of LTB_4. Thus, the EPO + H_2O_2 + halide system may regulate the concentrations of several leukotrienes during inflammation.

2. *Collagenase*

Because eosinophils are present during wound healing, it has been proposed that they are involved in tissue remodeling (207). Initial studies have shown eosinophil collagenase activity in chick and rat skin collagen (208). Subsequently, extracts of highly purified eosinophils from guinea pigs have been found to contain a metalloprotein able to degrade types I and III collagens (209). The collagenase activity requires addition of 4-aminophenylmercuric acetate for its expression. Another report has confirmed the activity of eosinophil collagenase on types I and III collagen, the two major connective tissue components of human lung parenchyma (210); no evidence of elastase or neutral protease activity has been found. The eosinophil collagenase is inhibited by ethylenediaminetetraacetate and not by phenylmethanesulfonylfluoride or α_1-antitrypsin. Thus, eosinophil collagenase is a classic vertebrate collagenase, namely, a metalloprotein cleaving collagen types I and III at approximately equal rates.

3. *Other Eosinophil-Associated Enzymes*

A variety of enzymes have been associated with the eosinophil including acetylcholinesterase, acid glycerophosphatase, adenosine triphosphatase, α-mannosidase, arylsulfatase, catalase, β-glucuronidase, nonspecific esterase, ribonuclease, cathepsin, acid and alkaline phosphatase, histaminase, phospholipase D, phospholipase B, serine:pyruvate aminotransferase, enoyl-CoA, 3-ketoacyl-CoA, and acyl-CoA oxidase (151,211–231). In addition, a phospholipid exchange protein has been reported to be preferentially associated with the eosinophil (56). The small granules of eosinophils contain arylsulfatase and acid phosphatase (17) which are held in a latent, inactive form (225). The subunit composition of arylsulfatase has recently been reported (230). Anti-eosinophil serum causes a suppression of the rise of phospholipase B activity which occurs during *T. spiralis* infection in keeping with the origin of this enzyme from the eosinophil (231).

In certain cases enzymes localized to eosinophil granules by immu-

noelectron microscopy have been found both on the granule matrix and crystalloid (220–224). Because the crystalloid is likely a single substance, it is difficult to reconcile the localization of many different substances to it. The possibility must be entertained that some localization may be nonspecific, although inspection of the above reports does not readily support this possibility. In recent studies MBP has been localized to the core of the human eosinophil granule, while EDN, EPO, and ECP are present in the matrix (82a,82b). Granules with well-defined electron-dense cores show localization of EDN and ECP over the matrix. In occasional granules apparent localization of EDN and ECP over the crystalloid has been noted; it is possible that a thin layer of electron-lucent matrix overlying the crystalloid may bind antibodies and falsely suggest localization to it.

D. Eicosanoids Produced by Eosinophils

Initial reports have shown that human and mouse eosinophils stimulated with calcium ionophore metabolize arachidonic acid predominantly through the lipoxygenase pathway with 15-hydroxyeicosatetraenoic acid (15-HETE) as the major product along with other 15-lipoxygenase products such as 5,15-di-HETE and 8,15-di-HETE and 5-lipoxygenase products (232,233). The ability of eosinophils to produce sulfidopeptide leukotrienes LTC_4 and LTD_4 was first shown in horse eosinophils (234,235) and shortly thereafter demonstrated in human eosinophils, both from normal persons and patients with eosinophilia (236–240). The quantities of LTC_4 produced by the eosinophil were increased when the EPO + H_2O_2 + halide system was inhibited (204) pointing to a mechanism for self-modulation of eosinophil LTC_4 production. Comparison of eosinophils and neutrophils has shown that eosinophils elaborate predominantly LTC_4 while neutrophils elaborate more LTB_4 (236,239,240). Interestingly, lipid bodies which are often present in eosinophils have been shown to concentrate [^{3}H]arachidonic acid and convert it to phospholipids (241). Thus, the lipid body may be a site for the processing of arachidonic acid in the eosinophil. The mast cell contains abundant lipid bodies (242). Finally, eosinophils have been triggered to release LTC_4 by exposure to IgG-coated Sepharose beads which, as discussed below, mimics the conditions causing eosinophil degranulation by worms (vide infra). In these latter experiments f-Met-Leu-Phe caused activation of eosinophils as judged by an increase in the amounts of LTC_4 released (243).

E. Other Eosinophil-Associated Substances

Sulfated complex carbohydrates have been identified in eosinophils by electron microscopy (244) and studies of eosinophil glycosaminoglycans have shown that chondroitin 4-sulfate predominates, 70–81%, with small amounts of chondroitin 6-sulfate, 9–12%, and dermatan sulfate, 5–12% (245).

Eosinophils are able to generate platelet-activating factor (1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) by an acetylation reaction involving the enzyme 1-alkyl-2-lyso-*sn*-glycero-3-phosphocholine:acetyl-CoA acetyltransferase (246). The activity of the enzyme in the isolated eosinophil has been stimulated by several chemotactic factors for eosinophils including C5a and ECF-A.

Eosinophils show marked autofluorescence, and they can be distinguished from other leukocytes by fluorescence microscopy (247). The autofluorescence of eosinophils is so great that it has been used as a means to purify eosinophils from peripheral blood of normal and eosinophilic donors by the fluorescence-activated cell sorter (248). The fluorescence intensity of blood eosinophils varies inversely with the logarithm of the donor's absolute eosinophil count. The chemical nature of the substance causing eosinophil autofluorescence is obscure.

IV. Light Density Eosinophils

As a consequence of efforts to obtain purified eosinophils, a population of eosinophils of lower than normal density has been found and referred to as light density or hypodense eosinophils (249–255). Initially, the existence of light density eosinophils complicated efforts to compare cells from normal individuals and patients with eosinophilia, in that the light density eosinophils were contaminated with neutrophils (249). Light density or hypodense eosinophils are present in the blood of patients with eosinophilia-associated diseases such as the hypereosinophilic syndrome, parasitism, allergy, and neoplasia and their numbers are positively correlated with the degree of blood eosinophilia (255). The density of normal eosinophils has been determined using polyvinylpyrrolidone-coated silica gel gradients (251,255,256); normal eosinophils have a peak density of 1.088 with fewer than 10% less than 1.082. In contrast, hypodense eosinophils have a peak density of 1.075–1.077. Light density eosinophils have reduced values of lactate dehydrogenase isoenzyme 5 compared to normodense eosinophils (254). Hypodense eosinophils possess greater numbers of IgG Fc receptors and complement receptors than do normodense cells (251); this finding should be reexamined in view of the earlier reports that complement receptors were not increased on eosinophils from patients with blood eosinophilia (30,35,39). In pleural fluids from patients with various lung diseases 80–100% of the eosinophils are hypodense, whereas in bronchoalveolar lavage fluids 40–70% are hypodense (251,253). The hypodense eosinophils show higher than normal oxygen consumption (251) and, most interestingly, show potent cytotoxic activity for antibody-coated targets (252). Indeed, IgE-dependent cytotoxicity for schistosomula of *S. mansoni* appears to be restricted to hypodense eosinophils (46). Thus, light density eosinophils are activated cells and may

represent the end result of the effects of the activating agents discussed in Section VII.

The existence of hypodense eosinophils may also explain in part prior observations of eosinophil heterogeneity. For example, striking differences in the ability of eosinophils from normal individuals and patients with eosinophilia to generate superoxide anion have been reported (257). Hypodense eosinophils show reduced numbers of granules (251–253), and they may have partially degranulated as a consequence of a prior activation step. Normodense eosinophils from patients with eosinophilia appear activated when compared to eosinophils from normal persons as assessed by their surface charge, activation of granule acid phosphatase, membrane hexose transport, and hexose monophosphate shunt activities (249); these activated cells could become hypodense. However, the possibility also exists that the hypodense eosinophils are released as such from the bone marrow.

The presence of light density eosinophils can reduce the yield of purified eosinophils by density gradient centrifugation because the hypodense eosinophils sediment with neutrophils (249,258). The recent finding that the chemotactic peptide, f-Met-Leu-Phe, causes a reduction in the density of neutrophils without affecting the density of eosinophils may provide a tool to increase yields during eosinophil purification. However, one must be alert to the possibility that f-Met-Leu-Phe activates eosinophils (243).

V. Eosinophil Degranulation

Interaction of eosinophils with IgG-coated schistosomula leads to degranulation as evidenced by release of granule MBP (106,109). However, investigation of the behavior of the eosinophil in this interaction is complicated by the presence of the viable worm. Therefore, model systems have been developed to study eosinophil degranulation using large surfaces coated with IgG. Initial investigations of Millipore filters as nonphagocytable surfaces (259,260) were abandoned when it was observed that eosinophils penetrated into the filter thus hampering ultrastructural studies (261). Similarly, use of collagen-coated coverslips was flawed by the ability of neutrophils to disrupt the collagen surface. Therefore, an agar layer has been used to coat coverslips, and it in turn is coated with IgG antibody and ECF-A (261). The behavior of eosinophils and neutrophils on this model surface has paralleled the interaction of these cell types with IgG-coated schistosomula. For example, eosinophils make more intimate contact with the surface than do neutrophils, and EPO and arylsulfatase are secreted by eosinophils (261); little or no cell lysis occurs during a 2-hour incubation period and the cytoplasmic enzyme lactic dehydrogenase is not released.

With the above system, plasma membrane changes during the interaction

of human eosinophils have been studied by membrane radiolabeling (262). A protein of 55,000 Da becomes newly accessible on the eosinophil surface during the early attachment phase of eosinophil interaction (263). The expression of this protein, termed protein 3, is enhanced by agents, such as ECF-A, which also enhance the expression of Fc receptors and, correspondingly, is inhibited by agents, such as hydrocortisone, which inhibit expression of Fc receptors (263).

A similar system has been established by exposure of eosinophils to Sephadex G-15 beads treated with human serum (264). Eosinophils release approximately 15% of their total content of ECP under these conditions. ECP release is inhibited by cytochalasin B and D and by hydrocortisone. No direct relationship has been found between degranulation and the respiratory burst; zymosan-treated serum and phorbol-12-myristate-13-acetate induce a respiratory burst but no ECP release (264). A variation of this method has been used to induce LTC_4 production by eosinophils (243) and to study eosinophil activation (265).

Eosinophil degranulation has also been studied by interference phase contrast microscopy (266). After exposure to staphylococci, eosinophil granules marginate along the plasma membrane and are discharged extracellularly. Degranulated cells are larger than resting cells, are amoeboid in shape, and exhibit large nude areas of cytoplasm. Pharmacological agents that increase intracellular cyclic adenosine monophosphate, such as isoproterenol, prevent degranulation. In this system endotoxin-activated serum, ECF-A, phytohemagglutinin, concanavalin A, levamisole, and compound 48/80 cause degranulation and disodium cromoglycate prevents degranulation (266).

Eosinophils have also been stimulated to degranulate with the calcium ionophore A23187 (267–270). Two studies of horse and human eosinophils showed noncytotoxic degranulation (267–270). In stimulated cells granules move to the periphery of the cell and adjacent granules fuse. The granules swell and their contents are released into large intracellular vacuoles which open to the outside. Degranulation of horse eosinophils, measured by EPO release, is blocked by eicosa-5,8,11,14-tetraynoic acid (which inhibits both cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism), but not by indomethacin (which only inhibits the cyclooxygenase pathway) (267). Morphometric analyses of human cells show a marked reduction in numbers of larger granules in the stimulated cells and an increase in numbers of small granules (270). Another study of human eosinophils has shown that A23187 causes degranulation, as evidenced by release of EDN, and this is associated with cell damage, as evidenced by LDH release (269). The concentration of A23187, 1 μg/ml, is below the toxic level, and degranulation is inhibited in the absence of Ca^{2+}. Neutrophils, purified from the same

donors, do not show evidence of damage when stimulated by the same concentrations of ionophore. Electron microscopic examination shows evidence of cytolysis; granule exocytosis was only observed once. Further work is needed to clarify the discrepancy between these studies (269,270) of the effects of A23187 on human eosinophils.

Finally, the contribution of the eosinophil to tissue damage has been examined by seeking evidence for eosinophil degranulation in diseased tissues through localization of granule proteins (vide infra). An ultrastructural correlate of MBP deposition may be loss of electron density in eosinophil granule cores (271). Eosinophils in diseased tissues have shown some granules with intact cores while other granules in the same cell have lost most of their cores. Although such a finding might be an artifact of fixation and embedding, it is difficult to envision such a random artifact. Thus, the finding of loss of electron density in the eosinophil may be an early sign of eosinophil activation and incipient degranulation. Another mark of eosinophil degranulation may be the occurrence of vacuolated eosinophils and cells with few granules (272).

VI. Phagocytosis by Eosinophils

Although eosinophils are phagocytic cells, they are less active than neutrophils (reviewed in 273). Early experiments have shown ingestion of immune complexes (274), and several studies have addressed this issue (275–278) by demonstrating requirements for IgG (277) or IgE antibody (276,278). Purified human eosinophils phagocytize ragweed-coated latex beads provided IgG-rich serum fractions are present (279). Studies of the phagocytic properties of eosinophils have, in general, been relatively neglected since the discovery that eosinophils are able to secrete granule proteins onto helminth targets and to kill these targets (280).

VII. Eosinophils as an Effector Cell Damaging Parasites

A. Evidence for an Effector Role for Eosinophils

By the early part of this century, the striking association between peripheral blood eosinophilia and helminth infection had been established (see 281 for summary). During the 1970s, an abundance of new evidence appeared supporting a role for the eosinophil as an effector cell in parasitism. This evidence consists of (1) the ability of anti-eosinophil serum (AES) to abolish immunity to helminths, (2) the direct killing of helminths by eosinophils and their products, and (3) the demonstration of eosinophil infiltration about and degranulation onto degenerating parasites.

1. *Effect of AES*

The effect of AES has been tested on animals infected with three helminths, *S. mansoni* (63,282), *T. spiralis* (64), and *Trichostrongylus colubriformis* (66), and a tick, *Amblyomma americanum* (91). Mice were infected by exposure to schistosomula of *S. mansoni*, and a specific anti-mouse eosinophil serum was administered before a rechallenge of schistosomula (63). The AES-treated animals had a striking reduction in the number of peripheral blood eosinophils and an associated loss of immunity as judged by the number of schistosomula isolated from the lungs. Eosinophil depletion by AES delayed damage to *S. mansoni* eggs (282); the suppression of egg destruction caused an increase in the severity of liver disease as judged by an increased portal pressure and splenic weight. AES administration to mice primarily infected with *T. spiralis* caused a marked increase in the number of encysted muscle larvae, but no change in the number of intestinal worms (64). This suggests that the eosinophil acts on newborn larvae or muscle larvae but not on intestinal worms. Guinea pigs have been treated with AES to determine whether susceptibility and immunity to *T. colubriformis* is altered (66). The results show a significant increase in susceptibility and a reduction in immunity. Finally, administration of AES and anti-basophil serum to guinea pigs exposed to the tick, *A. americanum*, shows that anti-basophil serum completely abolishes tick immunity and AES significantly reduces immunity to the tick (91). Both antisera have the expected effect on their respective targets in peripheral blood; eosinophils are ablated by AES and basophils by anti-basophil serum. However, in tick feeding sites AES reduces only the number of eosinophils, whereas anti-basophil serum causes a reduction in both basophils and eosinophils. The results suggest a role for the basophil in tick immunity and a cooperation between these cells in that immunity. In this cooperation basophils presumably attract eosinophils to the tissue sites and both participate in the attack on the tick (91).

2. *Killing of Parasites in Vitro*

Numerous studies have confirmed the first reports (283,284), showing that eosinophils damage schistosomula of *S. mansoni* (reviewed in 280). In this system IgG antibody coats the schistosomulum (37) and damage is correlated with the number of eosinophils (285). The attack on the parasite is associated with degranulation as shown by MBP release (106,109). Damage is altered by agents altering the cell surface membrane and microfilaments, but not by agents which inhibit DNA replication, protein synthesis, oxidative respiration, prostaglandin synthesis, and microtubule aggregation (286).

Eosinophil damage to schistosomula can be produced by binding eosino-

phils to the worm with concanavalin A and by using calcium ionophore to cause degranulation (109). The ability of the eosinophil to damage schistosomula has been shown using rat (38,287,288) and mouse (289) eosinophils. In the rat, C3 bound to schistosomula mediates eosinophil adherence (38,287), and activation of complement by the classical pathway enhances the antibody-dependent action of human eosinophils (290). Finally, schistosomula damaged by IgG antibody and eosinophils *in vitro* do not mature *in vivo* when injected into mice, thus justifying the use of the *in vitro* morphological assay as an estimate of irreversible damage (291).

Eosinophils also kill other parasites, including *T. spiralis* (292), trypanosomes (111–113,293–295), microfilariae (296,297), and flukes (298,299). Eosinophils from mice with the beige mutation (equivalent to the Chediak–Higashi syndrome in humans) show two populations of eosinophils, one with grossly enlarged granules and multiple crystalloids and the other resembling normal eosinophils (300). The normal eosinophils adhere to *T. spiralis* and degranulate, whereas cells with the large granules adhere loosely and do not degranulate. Despite the defect in their eosinophils, beige mice show a similar course of *T. spiralis* infection as normal mice (300).

Human IgG antibodies were first reported to mediate eosinophil killing of schistosomula (37). IgE antibodies are also active although IgE-dependent cytotoxicity may be restricted to hypodense eosinophils (46).

Rat IgG_a and IgE antibodies (44,288) including monoclonal IgG_{2a} (301) mediate eosinophil cytotoxicity, and mouse monoclonal IgE antibodies mediate killing of dinitrophenylated schistosomula of *S. mansoni* (302). The IgG_{2a}-mediated cytotoxicity may be mediated in part by soluble immune complexes bound to the eosinophil and thus acting as cytophilic antibody. Eosinophils from infected rats have directly killed nonopsonized schistosomula; immune complexes have been pelleted from serum by ultracentrifugation (303) and these immune complexes are able to induce killing by normal eosinophils. IgE antibody is present in immune rat serum after 6 weeks of infection, and mast cell products, especially ECF-A, appear to play an essential role in augmenting eosinophil cytotoxicity (44). IgG_{2a} and IgE mediate anaphylaxis in the rat; their identification as antibodies also mediating eosinophil cytotoxicity links the eosinophil to mechanisms occurring during anaphylaxis (304,305).

The observation that two cytophilic antibodies, IgG_{2a} and IgE, are prominent in the immune response to *S. mansoni* has prompted an inquiry into the role of the mast cell, with its high affinity receptor for IgE, in parasitism. IgE production is suppressed by neonatal injection of anti-IgE; IgE-suppressed rats do not produce antibodies of the IgE class, and when infested with *T. spiralis*, their mast cells do not release serotonin on exposure to antigens (306). Furthermore, the anti-IgE-treated rats have fewer tissue

eosinophils about nurse cells (muscle cells containing *T. spiralis* larvae), and greater numbers of larvae survive and encyst in muscle. This experiment supports a biological role for IgE, mast cells, and eosinophils in resistance to helminths. Interestingly, W/W^v mice which lack both connective tissue and mucosal mast cells expel adult *T. spiralis* more slowly than normal littermates (307).

Clinical studies have been conducted to define the characteristics of resistance in humans chronically exposed to cercarae of *S. mansoni*. Two studies did not find any striking differences between resistant and susceptible children other than an increase in the age of the resistant group (308,309). In particular, no differences have been found among eosinophil levels, IgE antibody levels to schistosomula, antibody levels to worm antigens or to adult worms, and levels of antibodies-mediating eosinophil-dependent killing of schistosomula. However, a more recent study of children living in an endemic area for *Schistosoma haematobium* found that exposure levels and peripheral blood eosinophil counts were important factors. Children who were reinfected had significantly higher levels of exposure and significantly lower peripheral blood eosinophil counts than children who were not reinfected (309a). Younger children may possess IgM and perhaps IgG-blocking antibodies which inhibit the activity of IgG antibodies mediating eosinophil-mediated worm killing (A. E. Butterworth and M. Capron, personal communication). Of interest is a possible difference (albeit not statistically significant) in the ability of mononuclear cells to activate eosinophils, with susceptible children showing less enhancement than resistant children (309). A related finding is the demonstration that mononuclear cells from *S. mansoni*-infected individuals elaborate less eosinophil cytotoxicity enhancing activity than noninfected persons in the same geographic region (310). Cell transfer studies in the rat have shown that the eosinophil is the major cell involved in early protection after infection and that IgG_1, IgG_{2a}, and IgE are present on its surface (311).

Recent experiments have shown that a rat IgG_{2c} monoclonal antibody to schistosomula is able to inhibit IgG_{2a}-dependent eosinophil-mediated cytotoxicity by competition for target antigens and for the eosinophil Fc receptor (312). The IgG_{2c} monoclonal antibody reacts with a 38,000 Da antigen on schistosomula and blocks the ability of eosinophil-bound IgG_{2a} antibodies to react with this antigen. Although the IgG_{2c} antibody apparently binds to the eosinophil, IgG_{2c} antibody does not mediate killing; rather, it inhibits the ability of eosinophil-bound IgG_{2a} to mediate killing, presumably by displacing IgG_{2a} from the eosinophil (312). During the early stages of rat infection (3–4 weeks), eosinophil receptors are occupied by cytophilic antibodies of the IgG_{2a} isotype and by IgG_{2c} thereafter (313). Inhibition experiments have shown that IgG_{2c} can, in fact, displace IgG_{2a}

from the eosinophils. These results demonstrate that IgG_{2a} and IgG_{2c} compete for a common receptor, and they illustrate a mechanism by which antibodies can modulate eosinophil effector function during schistosomiasis.

Although numerous studies have shown the ability of the eosinophil to kill schistosomula, the cytotoxic capability of the neutrophil has been more difficult to demonstrate. Direct comparison of the two cells indicated that the neutrophil was inactive (285,314), whereas other studies found that the neutrophil had a potent effect in the presence of antibody alone or more frequently with antibody and complement (290,315–320). Interestingly, EPO-coated schistosomules are more susceptible to killing by neutrophils (320); this behavior parallels the behavior of EPO-coated tumor cells (199). The reason for these differences remains obscure. Electron microscopic analysis of the adherence of eosinophils and neutrophils to schistosomula has shown that neutrophils fuse their membranes with the worm, whereas eosinophils adhere to the parasite surface through the discharged granular material and not by membrane fusion (321–323). Studies of killing of *T. spiralis* newborn larvae have shown that both eosinophils and neutrophils are active and comparable in their activities (324) and that newborn larvae have a remarkable susceptibility to H_2O_2 (325).

As noted earlier, degranulation of eosinophils appears to be an important event in the stable, essentially irreversible adherence of eosinophils to schistosomes (109). Phase-contrast (326) and electron (107) microscopy have shown that eosinophils degranulate onto the surface of schistosomula leaving electron-dense deposits on the surface of the organism. This process is associated with damage to the tegument of the worm in areas beneath the attached cells. The damage consists of an initial formation of blebs in the tegumental membrane followed by the destruction of the tegument. The released granule material contains peroxidase (287). Also, as noted earlier, eosinophils release MBP in concert with the attack on the organism (106,109). Finally, when incubated with MBP, schistosomula become swollen and lose their usual shape, and the tegument forms blebs and detaches (106,134). These changes are comparable to those seen with the direct eosinophil-mediated damage described above.

3. *Association of Tissue Eosinophils with Parasite Damage in Infected Tissues*

Eosinophils accumulate about parasites *in vivo* and deposit granule contents on them. For example, eosinophils have been found in close contact with the surface of schistosomula in the skin of monkeys immune to *S. japonicum* (327), and many of the challenge larvae have been destroyed in the skin at sites where eosinophils were numerous. Studies in other species have produced similar observations (328–331).

Recent observations indicate that eosinophils deposit toxic granule proteins on their targets *in vivo* (332). Skin biopsy specimens of patients with onchocerciasis have shown the presence of microfilariae of *Onchocerca volvulus* and eosinophils. During the Mazzotti reaction (an acute exacerbation of chronic onchocercal dermatitis characterized by intense pruritus, edema, erythema, and urticaria, and induced by treatment with diethylcarbamazine), immunofluorescent staining for MBP shows marked extracellular deposition of MBP surrounding degenerating microfilariae. Interestingly, the deposition of MBP is seen both in the presence and absence of eosinophil infiltrates. These observations suggest that eosinophils lose their morphological integrity during degranulation. If so, one must be cautious in the interpretation of pathology involving eosinophils; failure to see an eosinophil attached to a target does not mean that the eosinophil had not participated in its damage. Electron microscopic studies of lymph nodes from patients with onchocerciasis treated with diethylcarbamazine support this interpretation (333). Eosinophils adhere to microfilariae by flattening their membranes onto the parasite and they form an exact template of its surface topography. Eosinophils in direct contact with microfilariae die or show evidence of marked degeneration.

In murine toxocariasis, eosinophils in muscle granulomata show evidence of degeneration and appear to die (334). The association between worm damage and eosinophil adherence is variable, and eosinophils are not always adherent to worms even in sensitized animals (335).

Studies of blood eosinophils and serum from patients with filariasis after diethylcarbamazine treatment also provide insight into tissue events (336–338). For example, in patients with Bancroft's filariasis blood eosinophils decrease in number and serum MBP levels increase after treatment. The serum MBP elevation is correlated with the numbers of filariae in blood before treatment (336). These results suggest that eosinophils move into tissues from the blood and degranulate. Also, vacuolated eosinophils are present in the blood of patients with filariasis, and serum ECP is elevated suggesting that activation and degranulation of eosinophils have occurred (338).

B. Mechanisms of Damage to Parasites

As noted above, the eosinophil granule is a rich source of cationic proteins and evidence exists that four of these, MBP, ECP, EPX, and EPO + H_2O_2 + halide, damage helminths directly. Comparison of the toxicity of ECP and MBP for schistosomula indicates that ECP is about 10-fold more active than MBP on a molar basis (133,134). MBP (106,109) and EPO (287) are released during the attack on the worm's membrane. EPO kills schistosomula most efficiently in conjunction with H_2O_2 and halide (189). Eosinophils produce

H_2O_2 (183–185) when treated with various membrane stimulants (339) and when presented with antibody-coated schistosomula (317). Eosinophils and neutrophils do not differ in the capacity for H_2O_2 production (317). Eosinophils from two patients with chronic granulomatous disease, which were unable to mount a respiratory burst, had a diminished ability to kill schistosomula of S. *mansoni* compared to eosinophils from normal individuals, yet they were still active (317). Furthermore, antibody-dependent eosinophil killing of schistosomula occurs under anaerobic conditions (340). After 22 hours of anaerobic incubation conditions, 48.3% of worms are killed compared to 54.1% under aerobic conditions. This study supports the view that oxidative metabolism is not essential for eosinophil parasite killing and that cationic protein release is a major mechanism of antibody-dependent eosinophil helminthotoxicity.

C. Enhancement of Damage to Parasites: Eosinophil Activating Factor

Several factors enhance the eosiniphil's capacity to damage parasites. Lymph node cells stimulated with specific antigen or with mitogens produce a lymphokine which stimulates eosinophil migration. This factor, called the eosinophil stimulation promoter (ESP) (341), is heat stable, sensitive to proteases, and has an estimated molecular weight by gel filtration of 24,000–56,000 (342). Peripheral T cells produce ESP as judged by sensitivity to anti-θ and complement and by the absence of its production in nude mice (343). Tissue culture supernatants rich in ESP activity enhance the ability of eosinophils from normal mice to kill S. *mansoni* eggs *in vitro* (344). ESP causes a significant increase in the release of small granule arylsulfatase but not peroxidase (from the specific granules), and after 24 hours exposure ESP increases the hexose monophosphate shunt response to opsonized zymosan and phorbol myristate acetate (345). ESP action on eosinophils involves the generation of lipoxygenase products from arachidonic acid, and these regulate the migrating activities of murine eosinophils (346). Eosinophil localization at sites injected with ESP is dramatically inhibited by BW755C, which blocks both cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism (347).

Mast cells produce another series of factors which enhance the eosinophil's capacity to kill worms. Eosinophils incubated with the eosinophil chemotactic factor of anaphylaxis (ECF-A) show an increased number of complement (41,348) and Fc receptors (349) and an enhanced ability to kill schistosomula. The increase in Fc receptors is in apparent contradiction with an earlier report (vide supra) (41). Histamine increases the number of complement receptors (348,349) and stimulates superoxide production by eosinophils (350). ECF-A may mediate enhancement of eosinophil killing by

mast cell products noted earlier (349). Leukotriene B_4 from mast cells also enhances eosinophil killing albeit weakly (351).

Eosinophils from patients with eosinophilia possess an enhanced ability to kill schistosomula *in vitro* (352). This suggests that eosinophil colony-stimulating factors (CSF) might not only stimulate eosinophil production by the bone marrow, but also activate the eosinophil. Purified CSF-α both enhances eosinophil cytotoxicity and promotes eosinophil colony growth (353). CSF-α enhances the adherence step of the killing reaction; schistosomula are coated with several layers of eosinophils. The CSF-α enhancement of eosinophil adherence to schistosomules is temperature dependent and is not blocked by puromycin. CSF derived from human placentas contains CSF-α which stimulates eosinophilopoiesis and CSF-β which stimulates granulocyte–macrophage colonies. CSF-α stimulates both eosinophil and neutrophil killing of antibody-coated target cells, whereas CSF-β only enhances neutrophil-mediated killing of coated targets (354). CSF-α increases eosinophil autofluorescence and stimulates superoxide production, iodination, and protein synthesis by eosinophils (355). Thus, CSF-α acts at two sites: (1) on eosinophil progenitor cells where it stimulates differentiation, and (2) on mature eosinophils where it enhances functional activity. These findings are reminiscent of observations on recombinant granulocyte–macrophage CSF; this protein possesses colony-stimulating factor activity on neutrophil precursors, and its partial amino-terminal sequence is identical to that of neutrophil inhibitory factor which activates the mature neutrophil (reviewed in Ref. 356).

The finding that eosinophils from patients with eosinophilia have an enhanced ability to kill schistosomula *in vitro* has prompted a test of the role of mononuclear cells in eosinophil activation because eosinophilia is a thymus-dependent phenomenon (357–359). A factor has been discovered which enhances eosinophil antibody-dependent killing of schistosomula (360). This substance, derived from an adherent nonspecific esterase-positive mononuclear cell (and not a T cell), resists boiling, and has a molecular weight of 35,000–45,000 as judged by Sephadex G-200 fractionation. Because of the monocyte origin of this factor, it is probably not the eosinophil stimulation promoter which is produced by T cells (343). The monocyte factor is stable to dialysis, trypsin sensitive, and has a principal p*I* of 4.4 (361). It increases the proportion of eosinophils with Fc receptors, enhances the temperature-dependent phase of eosinophil adherence to antibody-coated schistosomula, and increases the uptake of deoxyglucose by eosinophils. This substance, now termed the eosinophil activating factor (EAF), has been partially purified from supernatants of cultured human peripheral blood mononuclear cells (362). It has a molecular weight of 40,000 ± 7,000, enhances the ability of eosinophils to lyse several targets, and increases the production of super-

oxide and H_2O_2 by eosinophils both spontaneously and after exposure to opsonized zymosan. However, the ability of EAF to enhance eosinophil killing evidently is not due to an increased respiratory burst because column fractions stimulating a respiratory burst are without effect on killing. Rather, the principal effect of EAF appears to be on eosinophil degranulation, occurring both spontaneously and after incubation with opsonized zymosan. Thus, EAF appears to enhance the capacity of eosinophils to kill parasites by augmenting degranulation and thus the amount of secreted toxic granule proteins.

Another monocyte derived factor, termed monocyte-derived eosinophil cytotoxicity enhancing factor (M-ECEF), has been produced by plastic-adherent cells, 91% peroxidase positive, from human peripheral blood (363). Whether this factor is the same as EAF is not clear at this writing. Comparison of the enhancing mechanisms of eosinophil killing by CSF-α and by monocyte factors (364) shows that both require oxygen and do not enhance killing under anaerobic conditions, that CSF-α increases superoxide anion production and leukocyte iodination (355), and that the monocyte factor has no effect on oxidative metabolism. U937 cells, a macrophage-like histiocytic cell line, and HL-60 cells, a promyelocytic leukemia cell line, produce factors enhancing eosinophil cytotoxicity (363). The M-ECEF from monocytes may be identical to the M-ECEF from U937 cells. M-ECEF from monocytes and U937 cells is heterogeneous with active fractions of molecular weight 17,000 and p*I*s of 4.8 and 5.1 (365). These properties resemble those of tumor necrosis factor which is also produced from monocytes (366) and U937 cells (367), has a molecular weight of about 17,000 (367), and p*I*s of 4.8–5.2 (368). Moreover, the molecular weight of tumor necrosis factor determined by gel filtration in physiological buffers is about 40,000 (369) similar to EAF (362). Because of these similarities, human tumor necrosis factor produced by recombinant DNA technology has been tested for its ability to enhance eosinophil killing (370). The factor has no direct effect on the parasites, but it enhances eosinophil toxicity to the worm in a dose-dependent manner and the effect is specific for eosinophils and not neutrophils. Moreover, tumor necrosis factor and M-ECEF coelute in reverse phase high-performance liquid chromatography and antibody to the tumor necrosis factor partially removes M-ECEF activity. The results show that tumor necrosis factor can enhance eosinophil function and may be an important regulatory molecule. Further work is needed to determine whether it is the principal factor in the monocyte supernatants or whether several monocyte-derived activating factors exist.

Supernatants from lymphocytes, both unstimulated and stimulated with mitogens and parasite antigens, enhance several eosinophil functions including hexose monophosphate shunt activity and chemotaxis (371). T lympho-

cytes stimulated with concanavalin A in the presence of 2-mercaptoethanol produce a factor enhancing eosinophil cytotoxicity (363). Rat T cell lines and clones specific for S. *mansoni* antigens have been established, and supernatants from these cells show a striking ability to enhance IgE and IgG_{2a}-dependent eosinophil-mediated killing of schistosomula *in vitro* (372). This enhancing activity is remarkably heat stable and survives 100°C for 10 minutes. T cell clone supernatants increase eosinophil degranulation as judged by peroxidase release (372). Thus, the known thymus dependency of eosinophilia has been extended to eosinophil activation and degranulation.

Finally, worm-derived factors may activate eosinophils. Schistosomula and homogenized 20-day-old worms and adult worms release a substance which activates eosinophils for IgG-dependent worm killing and increases IgG Fc receptors and degranulation of eosinophils (373). This substance is inhibited by the protease inhibitor, Trasylol, and after partial purification demonstrates neutral protease activity on collagenase substrates. Purified *Clostridum histolyticum* collagenase mimicks the enhancing effect of schistosomal proteases, suggesting the possibility that a collagenase-like enzyme is involved in eosinophil activation (374).

VIII. Eosinophils and Hypersensitivity Reactions

The results discussed above indicate that the eosinophil is well endowed with the machinery to destroy multicellular parasites. Furthermore, the eosinophil can be activated by numerous factors which enhance its destructive capability. These findings are in keeping with an inimical role for the eosinophil in hypersensitivity reactions. However, earlier findings have shown that the eosinophil possesses enzymes able to degrade mediators of anaphylaxis, and therefore, it has been regarded as a regulatory cell for control of immediate hypersensitivity reactions. Based on recent information, the view of the eosinophil as a regulatory cell in immediate type hypersensitivity reactions must be enlarged to include a role for the cell as an effector of tissue damage.

A. Modulation of Immediate Hypersensitivity Reactions

Eosinophil-associated enzymes have been shown to neutralize mediators of anaphylaxis, including slow reacting substance (375), histamine (216), and platelet activating factor (217). Therefore, it has been suggested that one function of the eosinophil is down-regulation of the inflammation following immediate-type hypersensitivity reactions (376).

Several additional supporting observations have been subsequently made. Human eosinophils, when stimulated by allergens or anti-IgE, liberate inhibitors of leukocyte histamine release, identified as prostaglandins E_1 and

E_2 (377,378). Furthermore, MBP binds heparin and neutralizes its anticoagulant activity (75), and EPO + H_2O_2 + halide degrades leukotrienes (204). Finally, eosinophils migrate to sites of IgE antibody interactions with many antigens, including those derived from parasites and pollen (327,379–382). Thus, eosinophils should be available to help regulate hypersensitivity reactions.

The hypothesis that the eosinophil modulates immediate hypersensitivity reactions, although attractive and eminently plausible, must be viewed with some caution because certain of the original bases of the hypothesis have not been confirmed, and because recent data have not fully supported the hypothesis. For example, it is now recognized that LTC_4, LTD_4, and LTE_4 account for the activity of the slow reacting substance of anaphylaxis (383,384). LTC_4 is an acidic lipid substituted at the 6-position with the tripeptide glutathione through a thiol–ether linkage. The absence of a sulfate ester suggests that LTC_4 is not susceptible to the hydrolytic action of arylsulfatase. Commercial arylsulfatase contains protease activity; this may have partially cleaved the glutathione moiety thus converting LTC_4 to the less active LTE_4 (385). When purified eosinophil arylsulfatase (230) was tested for its ability to inactivate the contraction of the guinea pig ileum by synthetic LTC_4 and LTD_4, the purified enzyme did not degrade the activity of the leukotrienes when tested at pH 5.7 or neutral pH (386,386a). Thus, the inactivation of SRS-A by eosinophil arylsulfatase B (375) and by arylsulfatases A and B from other sources (387,388) may be due to their contamination with dipeptidases, which convert LTD_4 to less active LTE_4 as assayed on the guinea pig iluem. The findings that eosinophils from patients with marked blood eosinophilia inactivate SRS-A are likely due to the ability of these activated eosinophils (251–253) to convert LTC_4 to the 6-*trans*-LTB_4 diastereoisomers and the *S*-diastereoisomeric LTC_4 sulfoxides through the generation of hypochlorous acid (204,236,389). Also, recent data suggest that eosinophil phospholipase D degrades a factor showing platelet lytic activity but not the platelet-activating factor (390). Finally, a direct test of the hypothesis that eosinophils inactivate mediators has been conducted using AES and glucocorticoids to ablate eosinophils from passively sensitized guinea pigs (67). These experiments have shown that neither ablation of eosinophils by administration of AES nor by methylprednisolone alters passive or systemic anaphylactic reactions when compared to controls. Tests of the effect of ablation of eosinophils on histamine release in the passively sensitized peritoneal cavity of the guinea pig actually show a reduction in histamine release. In contrast, histamine release following intraperitoneal injection of compound 48/80 is not affected by either AES or glucocorticoid administration, suggesting that stores of histamine are not depleted by these agents. This study concludes that the presence of eosinophils might actually contribute to histamine release.

B. Evidence for an Effector Role in Hypersensitivity Reactions

During the assault by eosinophils on a schistosomulum, the tegument is disrupted, showing the existence of powerful membrane active effector systems brought into action by degranulation. Presumably, MBP, ECP, and the EPO + H_2O_2 + halide system play a role in this damage. Because of the association between hypersensitivity diseases and eosinophilia (reviewed in Refs. 3 and 391) and because of the positive association between eosinophilia and organ dysfunction in bronchial asthma (392), the possibility that the eosinophil produces organ damage in bronchial asthma and in other hypersensitivity reactions has been tested.

Initial studies showing the toxic effects of MBP on murine tumor cells (106) prompted a test of guinea pig MBP on cells from organs that are infiltrated with eosinophils in disease (114). MBP damages skin, intestinal, splenic, and peripheral blood mononuclear cells at concentrations from 1×10^{-6} to 8×10^{-5} *M* in a dose-related manner. Cilia in tracheal explants are damaged as judged by absence of beating at about 10^{-5} *M* of MBP. Measurement of MBP in body fluids shows concentrations as high as 14 μg/ml, 1.5×10^{-6} *M*, in serum; 30 μg/ml, 3.2×10^{-6} *M*, in pleural fluid; and up to 93 μg/ml, 1×10^{-5} *M*, in sputa from patients with bronchial asthma. These results indicate that MBP is toxic to a variety of cells and that concentrations of MBP in the toxic range are present in human body fluids. In turn these findings suggest the hypothesis that the eosinophil might damage cells and impair organ function in disease states. This hypothesis is supported by prior observations that the eosinophil damages cells through antibody-dependent cell-mediated cytotoxicity (39), that eosinophils contain a neurotoxin (136), and that apparently degranulated eosinophils circulate in the blood of patients with the hypereosinophilic syndrome and associated cardiac disease (31). Similarly the finding of elevated levels of ECP has pointed to the possibility of a role for the eosinophil in rheumatoid inflammation (393,394).

C. Bronchial Asthma

A detailed analysis of the effect of MBP on respiratory epithelium from guinea pigs has shown that MBP at low doses produces exfoliation of epithelial cells and impairment of ciliary beating (395). In contrast, another basic protein, protamine, 1×10^{-4} *M*, produces neither exfoliation nor alteration of ciliary beating when incubated with tracheal rings for 72 hours. At the lowest concentration of MBP tested, 9×10^{-7} *M*, microscopic examination showed that the epithelium was disrupted and damaged cells were free in the lumen. With higher MBP concentrations, 50 μg/ml, 4.5×10^{-6} *M*, and 100 μg/ml, 9×10^{-6} *M*, the epithelium was extensively damaged

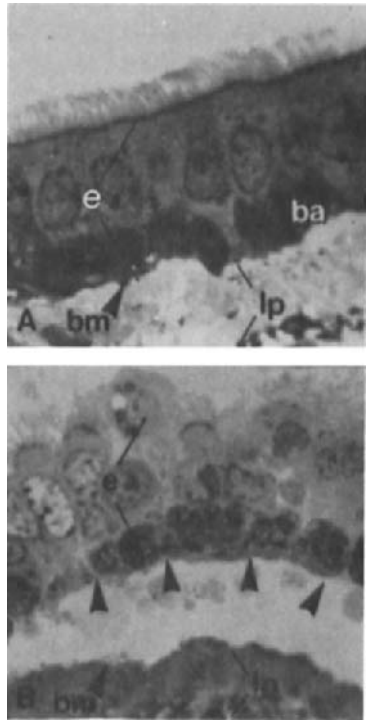

FIG. 7. Effect of human MBP on human bronchial epithelium. (A) Shows the control human bronchial ring after 68 hours in culture; note cilia (arrowhead) of surface epithelium (e), its basement membrane (bm), basal cells (ba), and lamina propria (lp). (B) Shows the human bronchial ring after treatment with human MBP, 100 μg/ml for 19 hours, the surface epithelium has detached from the lamina propria and damaged epithelial cells are seen free in lumen (arrowheads). ×600. (Photomicrographs generously provided by Dr. E. Frigas.)

with detachment of ciliated and brush cells and destruction of individual cells leaving only basal cells. Exfoliated cells were severely damaged, the cell membranes were lysed, and their cell contents liberated. These MBP effects on bronchial epithelium are reminiscent of the pathologic changes in human bronchial asthma. Shedding and desquamation of the bronchial epithelium down to the level of the lamina propria have been consistently reported in bronchial asthma (396–399). The superficial columnar cells become detached, a layer of basal cells remains behind, and regeneration of the mucosa presumably occurs from the basal cells.

The findings that MBP damages guinea pig respiratory tissue and that the damage mimics the pathology of asthma are consistent with the hypothesis that eosinophils, a hallmark of bronchial asthma, damage bronchial epithelium in asthma. Both human (Fig. 7) and guinea pig MBP damage respi-

ratory epithelium of both species indicating that MBP is not preferentially active on one or the other tissue (395,400). To determine whether MBP is present in sputa, it has been measured in the sera and sputa of patients with respiratory disease in general and asthma in particular. First, 100 sputa from patients with a variety of respiratory diseases were examined for MBP by radioimmunoassay (400). Sputa of 13 patients contained MBP levels greater than 0.1 μg/ml and 11 of these patients had asthma. Second, sputa of 15 patients hospitalized for asthma were analyzed sequentially for MBP content. Sputum MBP levels were elevated in all patients and rose from admission to day 3 before falling by day 8. The peak levels of sputum MBP in these 15 patients ranged from 0.3 to 92.9 μg/ml, geometric mean 7.1 μg/ml. These results indicate that elevated sputum MBP is a marker for bronchial asthma, and that high concentrations of MBP, in the toxic range, are present in sputa of many patients with asthma (400). Furthermore, sputa processed within a few minutes after expectoration contained high levels of MBP, suggesting that the elevation of MBP in sputa is not a consequence of *in vitro* cell death and release of granule MBP. In a subsequent study 116 sputa were analyzed and MBP levels were above 0.1 μg/ml in 39 (401). The 39 included 28 patients with asthma and 11 without asthma. Of the 11, 6 had chronic obstructive lung disease and pneumonitis, and the others had pneumonitis or neoplasm. Among the 77 with sputum MBP less than 0.1 μg/ml, none had asthma.

To test further the hypothesis that eosinophils mediate damage to bronchi in asthma, lung tissues from autopsies of patients dying of asthma have been examined for intracellular and extracellular MBP by immunofluorescence. An immunofluorescence procedure has been developed for MBP tissue localization such that MBP can be detected in formalin-fixed, paraffin-embedded tissues (402). Using this technique, lung tissues from 22 autopsy cases were studied (403). Sections stained with hematoxylin and eosin showed that all patients with asthma had a thickened basement membrane zone, goblet cell hyperplasia, and peribronchial inflammatory infiltrates containing eosinophils. Squamous metaplasia of bronchial epithelium, mucus plugging of bronchioles, Creola bodies, epithelial damage, and frank denudation of respiratory epithelium occurred with a more variable frequency. Immunofluorescence showed MBP staining which was intracellular as well as extracellular. The sites at which MBP was localized and the frequency of staining at these sites differed among the patients. Striking abnormalities were seen in patients dying with status asthmaticus, while patients dying of other causes with associated severe asthma showed abnormalities of a lesser degree. Patients dying of causes other than asthma showed neither epithelial damage nor extracellular MBP immunofluorescence. One pattern of MBP immunofluorescence which has been observed (Fig. 8) is diffuse staining of a

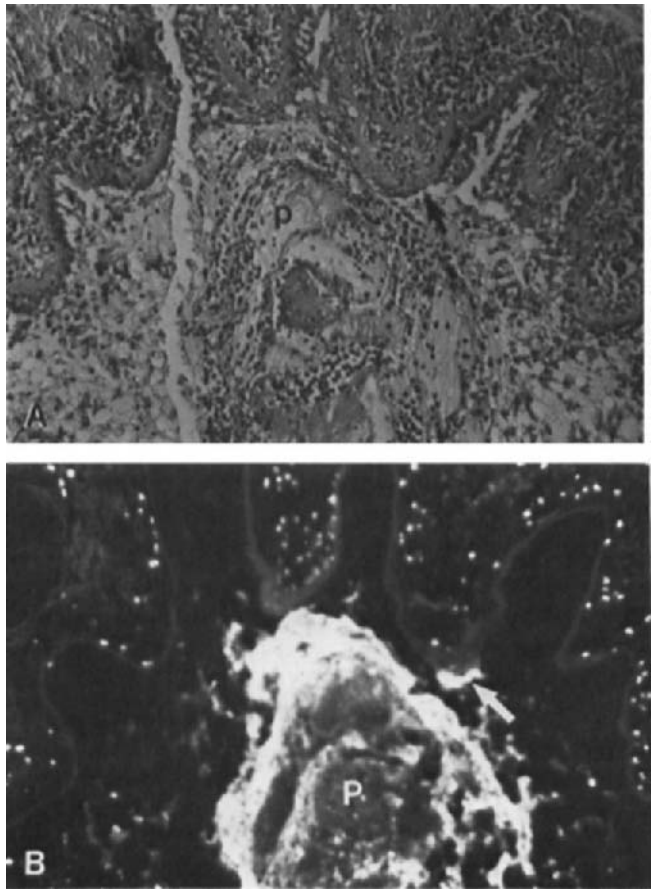

FIG. 8. Light microscopy and immunofluorescence microscopy of serial sections of bronchus from a patient who died in status asthmaticus. (A) Hematoxylin and eosin stain which shows basement membrane thickening, loss of respiratory epithelium (arrow), intense inflammatory infiltration of the lamina propria, occlusion of the bronchial lumen by a mucus plug (P), inflammatory cells, and cellular debris. (B) Immunofluorescence stain for MBP which shows MBP in mucus plug (P), MBP deposited on damaged epithelial surface (arrow), and discrete eosinophils below the basement membrane. ×100. [Used with permission from Filley *et al.* (403).]

mucus plug with brilliant glowing areas. The numbers of eosinophils infiltrating the mucus plug do not appear sufficient to explain the degree of fluorescent staining for MBP; presumably, this degree of staining reflects prior degranulation. In addition, the epithelial surface of this bronchus shows a loss of respiratory epithelium (Fig. 8A) which is congruent with an area of brilliant MBP immunofluorescence (Fig. 8B). A second immu-

nofluorescence pattern is diffuse extracellular MBP immunofluorescence in the lamina propria corresponding to the presence of necrotic, amorphous, eosinophilic material on the hematoxylin and eosin-stained section. Finally, a striking abnormality is intense extracellular MBP staining of damaged epithelium associated with eosinophil infiltration in the lamina propria and frank destruction of the basement membrane zone.

These results indicate that eosinophils degranulate in tissues and that a toxic granule protein is present at sites of tissue damage in asthma. Further, they suggest a mechanism for the chronic desquamation of bronchial epithelial cells which occurs in bronchial asthma. In this model eosinophils are attracted to the bronchial wall and lumen, either as a consequence of a preceding allergen–IgE antibody reaction with release of chemotactic factors culminating in a late phase reaction (404) as in allergic asthma, or through mechanisms evidently not involving IgE, as in intrinsic or idiopathic asthma. The recent finding that cytophilic IgE triggers eosinophil degranulation points to a mechanism by which eosinophils might degranulate on contact with allergen (49,50). As a consequence of eosinophil infiltration, toxic products, including both cationic proteins and potent oxidants from the reaction of EPO + H_2O_2 + halide, could damage the bronchial wall and epithelium, causing loss of bronchial epithelial cells. It is of interest that the participation of eosinophils in allergic asthma has been clearly anticipated in the description of guinea pig bronchial asthma in 1937 by Kallos and Pagel (405,406).

The finding that eosinophils preferentially produce LTC_4 (234–241), whereas neutrophils produce LTB_4 (236) suggests that eosinophils secrete this very potent smooth muscle spasmogen in asthma thus inducing bronchospasm. The ability of eosinophils to generate platelet-activating factor (246) indicates that they might also secrete this substance at sites of inflammation in the bronchial tree. Furthermore, rat mast cells stimulated with EPO + H_2O_2 + halide release histamine (193,196) and MBP activates purified human basophils and rat mast cells to release histamine (115–118). MBP is active at concentrations as low as 1×10^{-6} M, and MBP concentrations up to 10^{-5} M are present in sputum (400). Thus, eosinophils elaborate bronchospastic substances themselves, and they possess the means to stimulate basophils and mast cells to release histamine.

Recent findings support the possibility that eosinophils are a prime effector cell for tissue damage in bronchial asthma. Investigation of the local inflammatory process during late asthmatic reactions by bronchoalveolar lavage has shown a striking increase of eosinophils in the lavage fluids at the time of the late reaction (407). This bronchoalveolar eosinophilia is associated with an elevated ECP:albumin ratio in the lavage fluid, which suggests that eosinophils degranulate during the peak of the late reaction. A study of bronchoalveolar lavage in patients with mild allergic asthma (who did not

undergo antigen challenge) showed an increase in lymphocytes and eosinophils (408). A typical finding in these eosinophils is loss of the MBP-containing crystalline core as detected by electron microscopy. Comparison of the numbers of eosinophils in bronchoalveolar fluids from normal persons, patients with allergic bronchial asthma, and patients with asthma and idiosyncrasy to aspirin shows 0.4 ± 0.3, 3.9 ± 1.6, and $21.7 \pm 9.0\%$ eosinophils, respectively (409). These tissue-dwelling eosinophils are likely hypodense and therefore activated (251,252). Thus, eosinophils are present in the airways in asthma, and electron microscopy suggests they are in the process of degranulating. Ultrastructural examination of bronchial biopsies shows evidence of epithelial destruction in asthma (410). Ciliated cells appear to be the most damaged epithelial cell, and the epithelial destruction is extensive enough to expose epithelial nerves.

Serum ECP levels have been measured in patients with asthma and compared to normal individuals and patients with eosinophilia (411,412). Surprisingly, ECP levels are lower in patients with asthma than in patients with (corresponding degrees of) blood eosinophilia not associated with asthma. In one study bronchial challenge with antigen was associated with a decrease in serum ECP levels in one patient 5 hours after challenge (412); in another study serum ECP rose just after the maximal rise in airway resistance and fell to baseline levels in 3 to 5 hours (413). The peak in serum ECP following antigen inhalation in the latter study could be explained by eosinophil degranulation, possibly mediated by IgE (47,50). Further studies are needed to determine whether eosinophil degranulation occurs in the initial phases of allergic inflammation.

Two recent studies point to a possible relationship between the loss of bronchial epithelium in asthma and bronchial hyperreactivity. Studies of the effect of respiratory epithelium on the responsiveness of bronchial smooth muscle have indicated that removal of the epithelium increases the contractile responses evoked by acetylcholine, histamine, and 5-hydroxytryptamine (414,415). Transmural nerve stimulation causes similar peak contractions in muscle preparations with and without epithelium, but in the muscle with an intact epithelium a gradual decrease in the peak response has been observed (414). These results suggest that respiratory epithelial cells may generate a relaxant factor to decrease the responsiveness of bronchial smooth muscle to contractile agonists. The nature of the postulated factor is not known; initial studies suggest it is not a cyclooxygenase or lipoxygenase product of arachidonic acid metabolism (415). In asthma the postulated relaxant factor may be absent in areas where epithelial damage has occurred. Finally, in patients showing early and late asthmatic reactions after antigen challenge, the numbers of peripheral blood eosinophils are increased; in these patients, bronchial hyperreactivity to methacholine is significantly correlated with the

numbers of peripheral blood eosinophils (416). These findings relate the eosinophil to bronchial hyperreactivity, a stigma of asthma, and suggest that infiltrating eosinophils influence hyperreactivity, possibly by causing epithelial desquamation.

Eosinophils may contribute to a complication of asthma, the Churg–Strauss syndrome, and to other types of lung damage. Activated eosinophils are present in the granulomas of the Churg–Strauss syndrome, and both ECP and EPX are localized to cardiac and splenic lesions (417). Administration of polymyxin B aerosol to guinea pigs causes diffuse interstitial lung disease with alveolar wall thickening and alveolitis (418). Eosinophils are present in bronchoalveolar lavage fluids from treated animals and these eosinophils express cellular cytotoxicity to human lung fibroblasts. Finally, eosinophils and MBP also damage human and rat type II pneumocytes (419).

D. Cutaneous Disease

Several findings have linked the eosinophil to inflammatory cutaneous diseases associated with edema. In general, MBP is elevated in serum of patients with eosinophilia, and the degree of elevation is related to the magnitude of the blood eosinophilia (420). An exception to this rule are patients with chronic urticaria and atopic dermatitis who have elevated serum levels of MBP in the absence of peripheral blood eosinophilia. Tissues of these patients have now been studied by immunofluorescence for MBP localization (203,421). Surprisingly, eosinophil degranulation as judged by MBP deposition often occurs in the virtual absence of tissue eosinophils. In chronic urticaria extracellular MBP was present in 12 of 28 biopsies (43%); in contrast, disease controls including pemphigus vulgaris, ichthyosis vulgaris, actinic keratosis, basal cell carcinoma, and familial benign pemphigus did not show MBP outside of eosinophils (421). The MBP staining in chronic urticaria is of three types: (1) small blood vessel wall (5 patients), (2) dispersion of granular material (9 patients), and (3) focal or diffuse immunofluorescence of connective tissue fibers (11 patients). The finding that 43% of patients show evidence of tissue eosinophil degranulation (421) compares favorably with the prior finding that 38% of patients with chronic urticaria had elevated serum MBP (420). ECP has also been found outside of eosinophils in urticaria (128).

In atopic dermatitis, skin biopsy specimens from 18 patients has shown extracellular MBP deposition (203). MBP is deposited throughout the dermis in fibrillar and granular patterns. The fibrillar pattern corresponds quite closely to the pattern of elastic tissue fibers and suggests that MBP is deposited on elastic tissue. Onchocercal dermatitis is also pruritic and lichenified, and when stained for MBP, shows a pattern of extracellular deposition strikingly similar to atopic dermatitis. The mechanism involved in the deposition

of MBP in atopic dermatitis is unknown, but it could be a consequence of an IgE-mediated late phase reaction. Atopic dermatitis is associated with increased serum IgE concentrations, and in some cases, challenge with food allergens provokes the disease and causes an increase in plasma histamine levels (422,423). Preliminary studies of IgE-mediated late phase cutaneous reactions show a striking deposition of extracellular MBP (K. M. Leiferman, unpublished). Thus, the IgE-mediated late phase reaction is a likely cause of the deposition of MBP.

In addition to chronic urticaria, three other skin diseases associated with edema show evidence of eosinophil participation, namely Wells' syndrome, episodic angioedema associated with eosinophilia, and recurrent facial edema associated with eosinophilia. In Wells' syndrome, the eosinophilic cellulitis is associated with recurrent edematous and infiltrative plaques characterized by foci of amorphous eosinophilic material in the dermis, the characteristic "flame figures" (424). When examined for MBP by immunofluorescence, the flame figures show striking extracellular staining, suggesting that extensive eosinophil degranulation has occurred (425). Episodic angioedema associated with eosinophilia is characterized by recurrent attacks of angioedema, urticaria, and fever (426). During attacks, leukocyte counts increase to levels as high as 108,000 per mm^3 with up to 92% eosinophils and body weights increase up to 18%. The disease clearly waxes and wanes in concert with the number of eosinophils in the peripheral blood. By electron microscopy peripheral blood eosinophils show alteration of granules, and dermal eosinophils show a spectrum of abnormalities culminating in the frank destruction of the eosinophil and loss of its contents into the spaces among the collagen bundles. By immunofluorescence, MBP is present outside of the eosinophil around blood vessels and collagen bundles. Mast cells show evidence of degranulation by electron microscopy. Recurrent facial edema and eosinophilia is also associated with extracellular dermal MBP deposition and elevations of serum MBP (427). Thus, four cutaneous diseases show an association between eosinophilia and edema. The mechanisms underlying these diseases are incompletely understood, but the ability of eosinophils to elaborate LTC_4 and platelet activating factor and the ability of EPO + H_2O_2 + halide and MBP to cause histamine release from mast cells support the possibility that the eosinophil is a primary participant in the inflammation and edema associated with these diseases.

E. Cardiac Disease

The relationship between eosinophils and endomyocardial diseases has recently been reviewed (428–430). Studies over the past 8 years have shown that the presence of apparently degranulated eosinophils (31) in smears of blood containing greater than 1×10^9 eosinophils per liter is a simple

indicator of risk from cardiac disease. When this level of eosinophilia persists for weeks or longer, early acute endocardial lesions are invariably present (430). Why eosinophils localize to the heart is not known, but once there they deposit both MBP and ECP on the endocardium, about damaged cardiac myocytes, in mural thrombi and in the walls of blood vessels (430a). Supernatants from stimulated eosinophils are toxic to rat heart cells, and the toxicity is blocked by heparin, suggesting that the toxins are granule-derived basic proteins (431). The mechanisms by which eosinophils cause cardiac disease warrant further study, but the emerging picture suggests a role for toxic cationic proteins.

IX. Eosinophils and Reproduction

A considerable literature exists showing that eosinophils cyclically infiltrate the rodent uterus. Infiltration of the rat uterus by eosinophils, coincident with the estrus cycle (432), was observed during the 1950s; this observation has been confirmed and extended (433,434). Since then, various investigations have shown that (1) injection of estrogen into castrate or immature rodents causes an increase in uterine peroxidase activity (435) and a dramatic increase in uterine eosinophils (436), (2) the uterine eosinophil is marrow-derived (437), (3) uterine eosinophil numbers vary more than 100-fold during the normal estrus cycle (438), (4) eosinophils undergo lysis in the uterus around the time of estrus releasing their contents into the extracellular spaces (434,439), (5) eosinophils possess a cell-surface estrogen receptor (440), and (6) estrogen-induced uterine eosinophilia apparently has no dependence upon uterine mast cell activity (441).

The estrogen-induced uterine eosinophilia is associated with marked uterine edema (442) and it has been proposed that the eosinophil mediates the edema independent from genomic responses of the uterus itself to estrogen (443). A variety of conditions limiting uterine eosinophilia also block uterine edema. For example, glucocorticoids induce blood eosinopenia and prevent uterine eosinophilia and edema (444). Colchicine alters cell mobility by causing disaggregation of microtubules and blocks the development of uterine eosinophilia and edema after estrogen administration (445). Young rats have a relative blood eosinopenia, and in markedly eosinopenic animals estrogens do not induce either uterine eosinophilia or edema (446).

The mechanisms by which eosinophils might cause uterine edema are presently unknown; direct stimulation of vasopermeability by eosinophil LTC_4 (234–240) or indirect stimulation of vasopermeability by activation of mast cells by MBP (115) or EPO + H_2O_2 + halide (193) is obviously possible. Interestingly, eosinophils are associated with edema formation both in the uterus after estrogen administration and in the skin (421,424–427).

Finally, eosinophil migration to the uterus depends on the blood levels of estrogen rather than upon an estrogen-induced change in the uterus itself (447).

In contrast to the considerable literature on the presence of eosinophils in the rodent uterus, there are relatively few observations relating eosinophils to human reproductive physiology. Cyclic eosinopenia correlating with ovulation (448), and cyclic variations in endometrial eosinophils and their uptake of tritiated estradiol have been reported (449). Surprisingly, serum levels of immunoreactive MBP are elevated in all pregnant women (104). MBP levels increase during early gestation and plateau around 7500 ng/ml by the twentieth week (more than 10-fold above normal) before rising markedly just before the onset of labor. Levels return to normal following delivery, with a $t_{1/2}$ of 13.7 days. The MBP in pregnancy serum is remarkably similar to the eosinophil granule MBP by radioimmunoassay. However, no correlation between serum MBP level and peripheral blood eosinophil count exists in pregnant women, in contrast to previous studies of patients with eosinophilia (420). Also, levels of CLC, EDN, and ECP are normal or low in sera from pregnant women, whereas the serum levels of these proteins are elevated in patients with eosinophilia. Localization of immunoreactive MBP in placentas by immunofluorescence shows staining of anchoring villae, placental X-cells, and placental giant cells (105). Placental septal cysts stain brightly for MBP. Eosinophils cannot be demonstrated in the placenta. Septal cyst fluid contains up to 100 μg/ml MBP, a 6-fold greater concentration than the highest levels measured in maternal blood. These results support the hypothesis that an immunoreactive MBP is produced by the placenta. The function of the pregnancy MBP is presently obscure. Because the pregnancy MBP is present in placental-site giant cells which are located at the junction between maternal and placental tissues, one might hypothesize that the cytotoxic MBP plays a role in the invasive processes of early pregnancy. However, this hypothesis fails to account for the continued production of MBP throughout gestation, and for the marked late rise in MBP just prior to the onset of labor (104). Further work is needed to isolate the pregnancy MBP and investigate its properties and functions.

X. Summary and Conclusions

The evidence reviewed here indicates that the eosinophil has the ability to kill many species of helminths and likely does so during worm infection. This toxic ability appears to be regulated by several other cells including mast cells, monocytes, and T lymphocytes. Eosinophils kill helminths through their ability to generate potent oxidants and through their content of cationic proteins, which likely achieve high concentrations at points of granule deposition.

Eosinophils also participate in inflammation in human disease especially asthma, skin diseases, and heart disease. Though present concepts hold that the mast cell is the cornerstone of the allergic inflammatory response (450), the findings that eosinophils bind IgE and are activated by antigen–IgE complexes and that the eosiniphil can elaborate many inflammatory mediators raise the possibility that the eosinophil might also be involved in the initiation of inflammatory responses. Finally, an eosinophil-related protein appears to play an undefined role in human reproduction.

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ADVANCES IN IMMUNOLOGY, VOL. 39

Idiotypic Interactions in the Treatment of Human Diseases

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I. Introduction

The antigen specificity of the antibody is determined by the variable (V) sequences of the hundred or so amino acid residues at the amino-terminal end of each of the heavy (H) and light (L) polypeptide chains of the antibody molecule. The remaining constant amino acid sequences from the constant (C) domains determine the class of antibodies to which the molecule belongs.

Heterogeneity in the C domain distinguishes about 20 classes of immunoglobulin molecules, i.e., *isotypes* and distinguishes within each isotype a limited number of differences, i.e., *allotypes* which result from genetic polymorphism within the species. Heterogeneity in the V domain distinguishes millions of antibody molecules and forms the basis of antibody diversity. Because of this, antibody molecules can be immunogenic in genetically identical individuals, including the animal itself that produced these molecules. The antibodies elicited in these latter situations have been shown to be directed against antigenic determinants termed *idiotypes,* which are situated on the V domains of the immunizing molecules. Determinants recognized by antiidiotypic antibodies can be localized to either the variable heavy (V_H) or the variable light (V_L) polypeptides chain, but generally both chains contribute to the idiotypic determinants.

II. The Idiotype Network

In the network theory of regulation of the immune response proposed by Jerne, the immune system is regarded as a web of V domains based on interactions between idiotypes and antiidiotypes (Jerne, 1974). Antigen elicits the production of antibodies (antibody 1) bearing idiotypic determinants that are, in turn, able to induce the synthesis of antiidiotypic antibodies (antibody 2). The idiotypic determinants of these antiidiotypic antibodies can themselves elicit the production of other antiidiotypic antibodies (antibody

3). Before antigenic stimulation occurs, a steady state exists in which low concentrations of idiotypes are maintained. Antigenic challenge upsets idiotype equilibrium and perturbs the steady state, leading to increased production of antibodies (Ab1). These antibodies (Ab1) elicit the production of antiidiotypic antibodies (Ab2) resulting in the inhibition of the proliferation of progeny of clones (both B and T cells) initially stimulated by the antigen.

There is evidence to suggest that one characteristic of the idiotypic network is the occurrence of pairs of antibodies, i.e., of preferred partners. Thus, whereas the antibody response to foreign antigens is characterized by the production of several hundred different antibody molecules the anti-idiotypic response is much more restricted and favors the production of preferred complementary partners. A case in point is the anti-Id network related to the antibody response to the NP hapten in C57BL/6 mice. Injection of monoclonal anti-Ab2 gives rise predominantly to antibodies (Ab3) which are similar or identical to anti-NP antibodies (Ab1) rather than to a variety of antibodies which would be directed against different idiotypes and which would differ from Ab1 (Rajewsky and Takemori, 1983).

The above observation suggests that paratope–idiotype interactions are not vectorial in nature. In the vectorial concept of the network the concentration of immunoglobulin expressing a given idiotype (Ab1) is diminished by interacting with antibodies bearing complementary paratope (Ab2). In addition, the Ab2 by virtue of their own idiotype (i2) can stimulate the production of Ab3 which possess paratope (p3) specific for Ab2. Ab3 can use their idiotypes (i3) to stimulate Ab4, etc. This view of the network as open ended implies that the immune system is devoted to recognizing the idiotype rather than foreign antigens. However, immunochemical analysis of 4 members of an idiotype pathway obtained in the rabbit (Wikler *et al.*, 1979), and in the mouse (Bona *et al.*, 1981) has shown that in fact Ab1 and Ab3 share idiotopes, and both Ab2 and Ab4 bind to Ab1.

It has been postulated that Ab1 molecules express a special set of regulatory idiotypes (ri), as well as several conventional idiotypes (Bona, 1981; Paul and Bona, 1982). The central outcome of immunization with Ab1 is the production of anti-ri antibodies because only regulatory idiotypes would be immunogenic in a syngeneic and autologous system, whereas the "conventional" idiotopes are principally immunogenic across homologous and heterologous barriers. The Ab2 antibodies lack these regulatory idiotopes, but they are able to induce the synthesis of antibodies designated as Ab3, the majority of which share the regulatory idiotopes of Ab1 and only a few of which are true Ab3 (i.e., anti-Ab2 idiotopes). Consequently Ab4 antibodies obtained by immunization with Ab3 are principally anti-ri. This explains why Ab4 antibodies are found to bind to Ab3 as well as to Ab1 (Fig. 1). The regulatory idiotope concept predicts that the regulatory idiotopes expressed

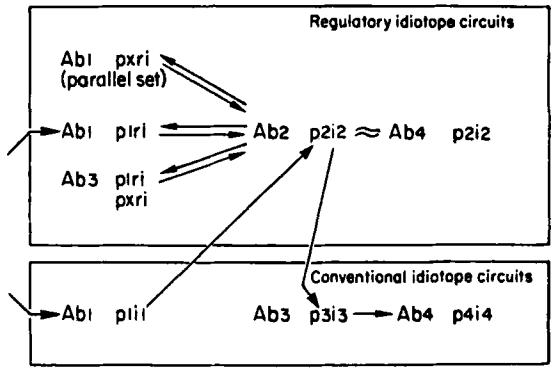

FIG. 1. A view of idiotope–paratope interactions based on the regulatory idiotope concept. Ab1, antiepitope antibody; Ab2, antiidiotype antibody; Ab3, anti-antiidiotype antibody; Ab4, anti-anti-antiidiotype antibody; p, paratope; i, idiotope; ri, regulatory idiotope; px, antibody with unknown specificity; p2, anti-ri paratope. (From *Immunol. Rev.* **79**, 31, 1984.)

on Ab1 (p1i1 = ri) are antigen noninhibited and can be shared with a majority of Ab3 antibodies, which are actually members of the Ab1 family (p1ri = true Ab1), as well as parallel sets (pxri). This was found to be indeed the case (Ollo *et al.*, 1981).

The idea that each paratope recognizes an idiotope obviously requires that conventional antigenic determinants expressed on foreign antigens (epitopes) must cross-react with idiotopes. Thus some idiotopes are the "internal images of the antigen." Anti-Id antibodies carrying the internal image of antigens are termed *homobodies* (Lindenmann, 1979). Studies on homobodies show that they can be used to stimulate clones which are already primed by antigen, idiotypes, or antiidiotypes (Rubinstein *et al.*, 1983).

Antiidiotype antibodies which have a high affinity for idiotypes, but also bind with low affinity to the Fcy fragment of human IgG, have been recently described and designated as *epibodies* (Manheimer *et al.* 1984; Chen *et al.* 1985). Such antibodies can have a regulatory effect on the immune response. They usually cause inhibition of antibody responses. This is in contrast to the enhancing effect of classical rheumatoid factors raised by lipopolysaccharide stimulation of mice.

III. T Cells Involved in Idiotype-Determined Regulation

There are several reports which clearly demonstrate the existence of regulatory T cell counterparts for Ab1 (Id) and Ab2 (anti-Id) antibodies. Helper, suppressor, or effector T cells specific for antigens can share the idiotypes of Ab1 (Bona, 1981). Idiotype-specific (Ab2-like) helper and suppressor T cells

can also be identified in naive animals (naturally occurring Ab2-like cells) (Bona and Paul, 1979) during an antibody response (Cerny and Caulfied, 1981), or after the parenteral administration of either idiotypes (Rubinstein *et al.*, 1983) or antiidiotypes (Owen and Nisonoff, 1978).

The existence of idiotype-recognizing helper T cells (ThId cells) is clearly demonstrated by the effect of their absence in several types of mice. Mice lacking ThId cells also lack B cells which secrete significant amounts of idiotype-bearing antibody. The helper activity can be reconstituted in these mice with a source of ThId cells. Thus, male offspring of (CBA/N × BALB/c)F_1 mice, which have a genetic defect in production of T15-bearing anti-PC antibodies, mice treated from birth with anti-μ chain antibodies, mice genetically deficient in T15 idiotype expression because they carry the Igh^b alleles, all display lack of T15-specific Th cells while having normal levels of conventional helper T cells (Bottomly, 1984). These ThId cells seem analogous to the helper cells specific for the Ig_{1b} allotype, defined several years ago by Herzenberg and co-workers (1976). Mice in which expression of Ig_{1b} is chronically suppressed, and mice genetically defective for expression of Ig_{1b}, lack these allotype-specific Th cells.

The function of ThId cells appears not to be MHC restricted (Bottomly and Mosier, 1981), or under the control of known *Ir* genes (Bottomly and Maurer, 1980). The role played by naturally occurring idiotype in the development of ThId is not clear. It does not appear that such cells use idiotype as a restriction element (analogous to Ia antigens) because there is no requirement that the antigen used to activate ThId cells associates in any way with idiotype-bearing immunoglobulin. MacNamara and Kohler (1984) have demonstrated that idiotype helper T cells recognize determinants on the H chain of Ab1. In addition they showed that these idiotype recognizing Th cells are primed by a first set of helper cells which recognize determinants on the L chain of Ab2.

There is a very large body of evidence demonstrating the role for suppressor T cells in the idiotypic network. Some of these suppressors are idiotype positive and secrete idiotype-bearing suppressor factors (Bona *et al.*, 1979; Weinberger *et al.*, 1979) (Nakanishi *et al.*, 1982; Cerny *et al.*, 1982). These cells and their factors may suppress the idiotype-positive antibody response by directly interacting with B cells or with idiotype-recognizing helper T cells (e.g., via an antigen bridge) causing their suppression. Antiidiotypic, i.e., idiotope recognizing suppressor T cells have also been described (Bona *et al.*, 1979; Rajewsky and Takemori, 1983; Wysocki and Sato, 1981). Again these cells may directly suppress idiotype-bearing B cells or inactivate helper T cells.

The mechanism of emergence of idiotype-positive and idiotype-recognizing helper and suppressor T cells is not clear. Thymic education of T cells

could be achieved through encounters with B cells and selection by B cell idiotypes (Martinez *et al.*, 1984; McNamara and Kohler, 1984). This hypothesis provides a satisfactory explanation for the apparent linkage of T cell specificities to B cell idiotypes, which is not based on a true genetic linkage. B cell idiotypes provide the internal images of antigen in the context of self; T cell clones are positively selected (educated) by these B cell internal images. Internal images, that T cells "see," appear to be individual Ig chains rather than intact Ig configuration (McNamara and Kohler, 1984). However, normally, free chains are not present in the circulation. Possibly antigen-presenting cells can separate Ig molecules into free polypeptides when they stimulate or educate T cell clones. The recognition of internal image or regulatory idiotopes by T cells as determinants on individual heavy or light chains has profound implications for the meaning of regulatory idiotopes. It is more economical to control the expression of evolutionary and physiologically related V_H genes within a given V_H family, directly via idiotopes on individual chains rather than to involve H–L-dependent idiotopes which would introduce restrictions in immune regulation based on H–L conformations. Furthermore assuming that regulatory idiotopes are expressed by all members of a V_H gene family, the expression of V_H genes of this family in the course of a specific immune response could provide protection against other threatening infections.

In the absence of V region sequence homology between B and T cells (Kronenberg *et al.*, 1983; Kraig *et al.*, 1983) the presence of idiotypic or idiotype recognizing T cells probably represents a homology between three-dimensional conjugates of determinants on antigen receptors of B and T cells. In this fashion the T cell system appears to be an independent system, like the nervous system, which is related idiotypically to the B cell system (Ertl *et al.*, 1982). In as much as the reactivity of nerve cells with anti-Id (Ab2) to reovirus represents structural similarity, rather than primary amino acid sequence similarity, between B cell idiotype and nerve cell virus receptors, the reactivity of T cells with antiidiotypic antibody is the result of sharing of structural configuration rather than of *V* region gene determined sequence between B and T cell receptors.

The naturally existing balance maintained between the clones of the immune system based on fragile idiotype links can be perturbed by antigen, idiotype, and antiidiotype. The nature of the perturbation achieved with each of these agents, e.g., enhancement or suppression, depends to some extent on the dose administered and on the age of the recipient. Administration of antiidiotypic antibodies at high doses to adults or newborn mice generally suppresses the immune response via activation of idiotype-specific suppressor T cells. In contrast administration of minute amounts of the same antiidiotypic antibodies to neonatal mice activates an idiotype-positive im-

mune response via direct interaction of the antibodies with idiotype-positive B cells (Bona *et al.*, 1984). A similar activation of an idiotype-positive response can be achieved in neonatal mice by injection of monoclonal idiotype-bearing antibodies (Rubinstein *et al.*, 1984). These elicit idiotype-specific helper T cells. The physiological relevance of "idiotype-driven" expansion of the repertoire relates to the effect of transplacentally transferred maternal antibodies on the immune response of the neonate. Influx of maternal antibodies into the fetus has been shown to exert a profound effect on the development of the immune repertoire. Antibodies expressing a particular idiotype can indirectly expand idiotype-bearing B cells by first stimulating idiotype-specific helper cells. This maternally induced idiotype expansion in the neonate represents a natural priming of clones which would allow a more prompt and vigorous response against pathogens relevant in the species thus conferring a survival advantage to that species.

The following simplified sequence of events could occur in the suppression of the idiotype-positive antibody response (Ab1) (Fig. 2). Antigen induces the synthesis of antibody 1 (idiotype). When the level of idiotype

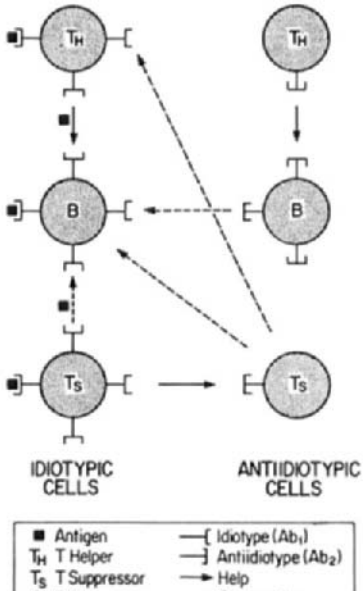

FIG. 2. Idiotype–antiidiotypic interactions. Only some of the interactions that occur at the level of the idiotype-positive B cells and affect idiotype (Ab1) synthesis are illustrated. Interactions can occur between sets of cells bearing complementary idiotypic determinants, or between sets of cells bearing identical idiotypic determinants through either an antigen bridge or complementary idiotype bridge. In this simplified model only Ab1 and Ab2 are shown.

attains a certain threshold it causes idiotype-specific B cells to produce antiidiotypic antibody (Ab2). The resulting antiidiotypic antibody (Ab2) can modulate, with or without the participation of complement, the function of idiotype-bearing B cells and T cells. Thus, idiotype-bearing antigen-specific suppressor T cells can be triggered to secrete idiotype-positive suppressor factors, which can then trigger a second subset of suppressor T cells that bear antiidiotypic receptors. This latter cell can then recognize and suppress idiotype-bearing B cells either directly or through inactivation of anti-idiotype-bearing helper T cells. As the level of antiidiotypic antibody (Ab2) rises, it triggers the production of anti-antiidiotypic antibodies (Ab3), which can inactivate the antiidiotypic suppressor cell and thus stop suppression of idiotype (Ab1) synthesis. Such waves of idiotypic regulation occur until a new steady state is reached. Suppression has been the predominantly observed regulatory pattern of the network especially in the case of auto-antiidiotypes (Rowley *et al.*, 1973; Schrater *et al.*, 1979). Enhancement has been, however, observed when anti-Id from another animal or a different species has been injected prior to antigen (Eichmann and Rajewsky, 1974). When antigen is introduced enhanced production of the relevant idiotype is observed. This has potential applications for immunization as discussed below.

IV. Regulation of the Normal Human Immune Response by Idiotypic Interactions

A. Anti-Id Antibodies to Human Anti-TT Antibodies

In the past few years evidence has been gathered to indicate that anti-idiotypic antibodies can regulate the human immune response to antigen *in vitro* and possibly *in vivo*. We will discuss data obtained with heterologous anti-Id antibodies as well as with autologous anti-Id antibodies in the tetanus toxoid (TT) system extensively studied in our laboratory.

Rabbit anti-Id antibodies have been raised against $F(ab')_2$ fragments of IgG anti-TT and rendered idiotype specific following appropriate absorptions with autologous TT nonreactive IgG $F(ab')_2$ and with pooled IgG (Geha, 1982a–c, 1984). These antisera precipitated from 30 to 50% of radiolabeled immunogen, i.e., ^{125}I-labeled $F(ab')_2$ anti-TT (Fig. 3). This was not surprising because the human IgG anti-TT response is a heterogeneous one. Thus, idiotypic determinants which are expressed on antibody molecules which are present in very small amounts may not be able to elicit an antibody response. The anti-Id antisera obtained always exhibited vigorous reactivity with sites related to antigen binding because (1) TT antigen vigorously inhibited the binding of ^{125}I idiotype to anti-Id (Fig. 4) and (2) because the

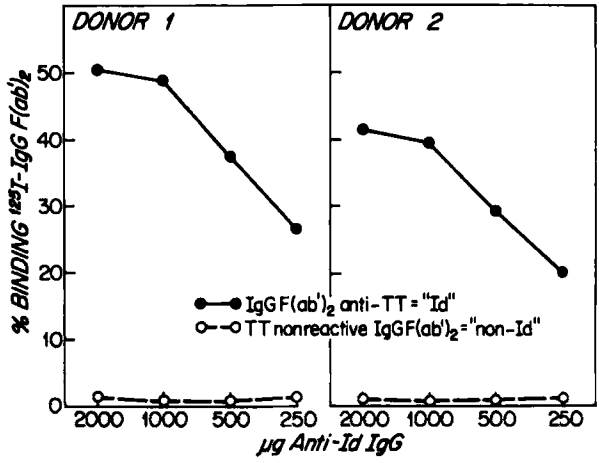

FIG. 3. Idiotype binding by rabbit antiidiotypic IgG. Approximately 50 ng of ^{125}I-labeled IgG F(ab')$_2$ anti-TT "idiotype" and of TT nonreactive IgG F(ab')$_2$ "nonidiotype" was added to various amounts of rabbit anti-IgG raised against the "idiotype" of the individual subject. The percentage bound was calculated as the ratio of the counts per minute (cpm) precipitated by the anti-Id to the cpm precipitated by rabbit anti-human Fab × 100.

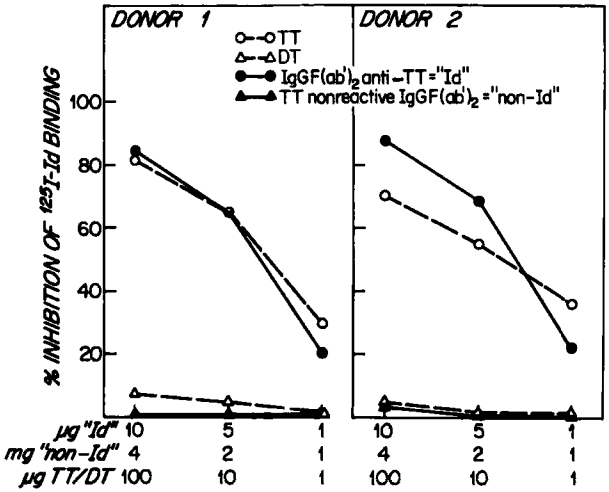

FIG. 4. Inhibition of ^{125}I-labeled "idiotype" binding to anti-Id IgG (500 μg) by "idiotype," TT, "nonidiotype," and DT.

anti-Id inhibited the binding of TT antigen to IgG. The anti-Id antibodies obtained showed poor or little cross-reactivity with IgG anti-TT from unrelated donors. Because in our initial studies we have absorbed the anti-Id antisera with pooled IgG which contained anti-TT we may have removed antibodies which recognized cross-reactive idiotypes (CRI). We have recently reexamined the presence of CRI using as probes anti-Id antisera which were carefully absorbed with pooled human IgG which was depleted of reactivity TT antigen. The resulting antisera exhibited variable and weak cross-reactivity with IgG anti-TT idiotypes from donors unrelated to the source of the immunizing idiotype. Cross-reactivity ranged from 0 to 20% reactivity of individual antisera of control and averaged from 5 to 10% of control reactivity with various antisera as determined by several assays including (1) direct idiotype of unrelated idiotypes, (2) competitive inhibition of the binding of the immunizing idiotype to anti-Id by unrelated Id, and (3) inhibition by anti-Id of TT binding to IgG from unrelated donors.

The relatively small degree of idiotypic cross-reactivity of IgG anti-TT idiotypes among unrelated subjects in the IgG anti-TT system is in agreement with the observations of other investigators who studied cross-reactive idiotypes on antibodies to foreign proteins from healthy subjects. Altevogt and Wigzell (1983) used rabbit anti-Id serum raised against IgG $F(ab')_2$ anti-TT from a single donor and found that only one out of four unrelated donors tested showed cross-reactivity. Pasquali *et al.* (1980) analyzed idiotypic cross-reactivity on IgM rheumatoid factor (RF). Rabbit anti-Id to IgM-FR from a patient with rheumatoid arthritis reacted with the patient's RF but not with 10 of 11 polyclonal and monoclonal RF from unrelated individuals. In their study of anti-Rh antibodies Navig *et al.* (1976) could detect CRI in only 2 out of 22 subjects. The results of these studies contrast with those of Cheung *et al.* (1985) who demonstrated CRI on anticasein antibodies of 12 out of 16 IgA-deficient patients detected by a rabbit anti-Id to anticasein IgG from an index patient. The reason for the high prevalence of CRI in these patients is not clear. It is possibly related to the nature of the antigen studied. In contrast to TT, a high-molecular-weight bacterial protein, casein, is a family of proteins also present in humans which are of varying molecular weights (12,000–24,000) and therefore may present a limited number of immunogenic sites resulting in restricted antibody heterogeneity. This factor may be one reason why autoantibodies tend to share cross-reactive determinants (see below). It is also possible that certain V restrictions are linked to IgA deficiency rendering IgA-deficient patients more likely to share certain variable region genes, e.g., those used in anticasein antibodies than normal subjects. Finally repeated immunization of IgA-deficient patients with casein via their gastrointestinal tracts may have resulted in the selective predominance of anticasein antibodies with CRI. Predominance of CRI is

known to occur after repeated immunization in mice (Conger *et al.*, 1981). In this regard Bose *et al.* (1984) have presented evidence for CRI in patients repeatedly immunized with ragweed antigen in the course of allergic immunotherapy and Saxon and Barnett (1984) have presented evidence for CRI at the T cell level in subjects repeatedly and vigorously hyperimmunized with TT antigen. It should be noted that in the latter two studies as well as in the anticasein study the contribution of anti-Id antibodies bearing the "internal image" of antigen has not been assessed.

Studies on cross-reactive idiotypes of anti-TT antibodies in two families showed that idiotypic cross-reactivity among members was significantly higher than among unrelated subjects. The exact interpretation of CRI among family members is difficult in the absence of primary sequence data. Idiotypic antigens are serologic markers which indirectly identify portions of the variable regions of immunoglobulin light and heavy chains. A central question is whether the idiotypic cross-reactivity observed by serologic methods will be found to be due to similar amino acid *V* region sequences. In most mouse systems, this has been found to be true. In some instances a substitution of one amino acid has resulted in major changes in the profile of idiotypic reactivity (Redbrugh *et al.*, 1985). For human proteins, the *V* regions of rheumatoid factors of the PO (for Pompa protein) or La (for Lay) proteins are homologous and IgM anti-DNA molecules isolated from sera of different patients with systemic lupus erythematosus also have similar sequences (Kunkel, 1984). There are, however, exceptions in which idiotypic cross-reactivity of murine antibodies has been found to be due to very small regions, or single amino acids, carbohydrate groups, or no sequence homology at all. It seems unlikely, however, that similar variable regions as detected by our anti-Id antisera could be generated in family members exclusively by somatic diversification. The results rather suggest the inheritance of antibody genes related to the idiotypic determinants in humans.

B. Binding of Anti-Id to Human B and T Cells

Rabbit anti-Id IgG was found to bind to 4–10/1000 human B cells (Geha, 1982c). The binding was completely inhibited by cold "idiotype" and partially but specifically inhibited by TT antigen. This latter observation suggests that the anti-Id recognized determinants that were related as well as determinants that were unrelated to the antigen binding site. The idiotypic determinants detected on the B cell surface were shown to be synthesized by the B cells because pulsing the B cells with radiolabeled amino acids followed by immunoprecipitation of cell surface proteins with anti-Id resulted in the precipitation of material which comigrated on SDS gels with immunoglobulin. Preclearing the cell lysate with anti-Ig resulted in the loss of the ability of the anti-Id to precipitate radiolabeled material from the B cells.

Rabbit anti-Id was also capable of binding to human T cells. The frequency of Id + T cells was very low. It ranged from 2 to 4/1000 T cells (Geha, 1984). The rabbit antiidiotypic antibody bound to T cells and not to B cells that contaminated the T cell preparations. Three lines of evidence argued in favor of binding to T cells. First, the frequency of B cells that bind to the rabbit anti-Id (anti-TT) was less than 1%. Because the T cell preparations contained less than 1% B cells, idiotype-positive B cells in these preparations would occur at a frequency of less than 1 per 10,000 cells. This is much lower than the observed frequency of idiotype-positive cells (24 to 38 per 10,000) in the T cell-rich preparations. Second, capping of cell surface immunoglobulins on 70% of B cells by preincubation with GAHIG, a polyclonal goat anti-human immunoglobulin, resulted in a negligible diminution (<15%) in the frequency of idiotype-positive cells detected in the T cell preparations. Third, double immunofluorescence staining revealed that less than 10% of the ID^+ cells had surface Ig.

The binding of rabbit anti-Id to T cells was idiotype specific. It was inhibited by the IgG anti-TT used for immunization and by TT antigen, but not by TT nonreactive IgG nor by DT antigen. TT was less efficient than IgG $F(ab')_2$ anti-TT in inhibiting anti-Id binding to the T cells. This phenomenon was previously seen with B cells and may reflect the presence of shared idiotypes on receptor molecules that differ in their specificity for antigen (Oudin and Cazenave, 1971; Eichmann *et al.*, 1977; Wysocki and Sato, 1981).

The partial, rather than complete, inhibition of anti-Id binding to T cells by IgG anti-TT contrasts with the complete inhibition seen in the case of the B cells. It is possible that after its injection into the rabbit, IgG $F(ab')_2$ anti-TT dissociated into H and L chain that then induced anti-V_H and anti-V_L antibodies. Either of these classes of antibodies could potentially react with T cells, and not with B cells. Such a reaction would not be inhibited by undissociated IgG $F(ab')_2$ anti-TT. In this regard, expression of V_H-related idiotypes, rather than V_H + V_L idiotypes by T cells has been reported (Krawinkel *et al.*, 1977; Chanh and Cooper, 1983).

The reactivity of anti-Id antisera with T cell suggested that the idiotypic determinants expressed by T cells were shared with those expressed by B cells and by serum antibody. It is possible, however, that circulating T cell-derived antigen-specific material copurified with the IgG anti-TT used to raise the anti-Id as suggested by the work of Iverson *et al.* (1983) and Cone *et al.* (1981). If that was the case then the rabbit antiserum could have contained both anti-T cell Id reactivity and anti-B cell Id reactivity, and these two reactivities could be directed against unrelated determinants. Two lines of evidence argue against the presence of antibodies directed against T cell-derived antigen-specific material in the rabbit antiserum. First, the IgG $F(ab')_2$ anti-TT used for immunization of the rabbits resolved in only one

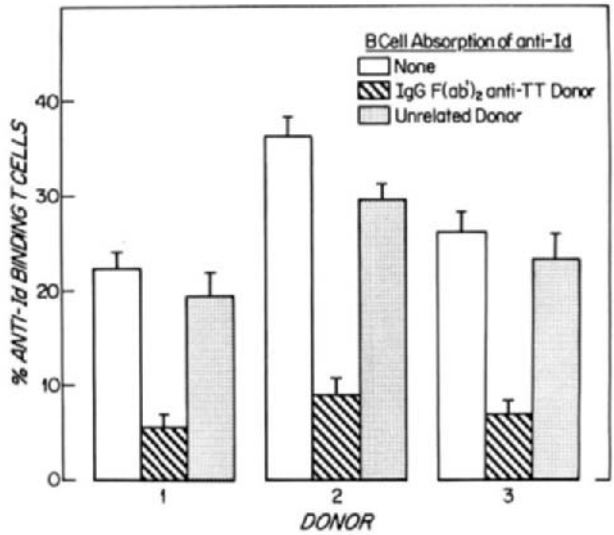

FIG. 5. Effect of adsorption of anti-Id antisera on their reactivity with T cells. The antisera were absorbed with no cells, EBV-B cells derived from the IgG $F(ab')_2$ anti-TT donor, or EBV-B cells derived from an unrelated donor. The antisera were then tested by indirect immunofluorescence for their capacity to specifically bind to T cells from the IgG $F(ab')_2$ anti-TT donor. The number of fluorescent T cells is shown on the ordinate; the donor tested is shown on the abscissa. (From *J. Immunol.* **133**, 1848, 1984.)

band on PAGE. Second, absorption with B cells derived from the IgG $F(ab')_2$ anti-TT donors, but not with B cells from an unrelated donor, removed the reactivity of the anti-Id antiserum with T cells (Fig. 5).

In experimental animals there is strong evidence to suggest that B and T cells share idiotypic determinants because polyclonal as well as some monoclonal antibodies to highly purified myeloma proteins with known antigenic specificity bind to suppressor T cells and to supressor T cell hybridomas and their soluble factors (Nakanishi *et al.*, 1982; Cerny *et al.*, 1982). Sharing of idiotypic determinants between T and B cells, however, does not imply structural similarity of their antigen receptor at the primary amino acid sequence level but simply implies similarity in the three-dimensional structure of their antigen receptors. Indeed, it is now abundantly clear that Id^+ T cells do not express V_H gene products (Nakanishi *et al.*, 1982; Kraig *et al.*, 1983; Kronenberg *et al.*, 1983). Precedents for cross-reactivity of antibodies with molecules that have different primary amino acid structures are well known: a case in point is the cross-reactivity of antiidiotypic antibodies to antireovirus 3 hemagglutinin with the receptor for the hemagglutinin expressed on somatic cells (Nepom *et al.*, 1982).

Idiotypic determinants were expressed on $T8^+$ cells but not on $T4^+$ cells

TABLE I
DISTRIBUTION OF T CELLS SPECIFICALLY BINDING RABBIT ANTI-Id[a]

T cells	Subject 1	Subject 2	Subject 3
Unfractionated	28[b]	37	30
T4+	3	3	4
T8+	61	90	87

[a] The frequency of positive cells represents the difference between the frequency of cells binding FITC-GARIG after preincubation with rabbit anti-Id IgG $F(ab')_2$ minus the frequency of cells binding FITC-GARIG after preincubation with rabbit preimmune IgG $F(ab')_2$. The latter never exceeded 4 per 10,000.

[b] Immunofluorescent cells per 10,000 cells.

(Table I). T8+ cells have been reported to bind directly antigen (Lehner, 1983) and anti-V_H antibodies and to inhibit antigen binding to human T cells (Lea and Michaelsen, 1982). T8+ cells contain predominantly suppressor and cytotoxic cells. Both types of cells have been shown in experimental animals to express idiotypic determinants (Weinberger *et al.*, 1980; Cerny *et al.*, 1982; Nepom *et al.*, 1982).

C. Effect of Heterologous Anti-Id on the Human Immune Response

Rabbit anti-Id antibodies to human IgG anti-TT have demonstrable effect on the TT-specific immune response by B and T cells *in vitro*. Anti-Id IgG was found to inhibit the pokeweed mitogen (PWM)-driven production of IgG anti-TT antibodies by peripheral blood mononuclear cells (Geha, 1982a). Anti-Id also inhibited IgG anti-TT antibody production in B cells stimulated with supernatants of PWM-stimulated T cells demonstrating that anti-Id can exert direct inhibitory effects on B cells. Anti-Id IgG by itself induced no Ig synthesis in B cells (Geha, 1979) even upon addition of T cell supernatants known to contain BCGF and BCDF activity. However when anti-Id antibodies were digested with pepsin and their fragments were used to stimulate B cells, there was induction of IgG anti-TT synthesis upon addition of BCGF/BCDF containing supernatants (Brozek *et al.*, unpublished observations). These results demonstrate that anti-Id antibodies can have opposite modulatory influences on human B cells and suggest that they can play a role in the regulation of the human immune response.

In addition to the effect of anti-Id on B cells anti-Id had demonstrated effects on the antigen-specific immune T cell response (Geha, 1984). Anti-Id

by itself caused a modest but consistent proliferation of T cells with stimulation indices of 2–3× the control. The major effect of anti-Id on T cells was that it induced substantial antigen-specific suppressor cell activity. The targets of this suppression were antigen-induced T cell proliferation and antigen-driven helper T cell-dependent antibody synthesis by B cells. In the case of proliferation the target of suppression would be a T cell, although a role for the monocytes in the delivery of the suppressor signals cannot be excluded. In the case of antigen-driven antibody synthesis, T and B cells could have been the targets of suppression.

When anti-Id antibody was directly added to cell cultures at the same time as antigen, no suppression was evident. Suppression was seen when T cells were preincubated for 48 hours with the anti-Id antibody and then added to resting cells. These observations suggest that there is a lag period for the expression of suppressor activity by T cells that interact with anti-Id. In cultures receiving TT and anti-Id simultaneously, helper T cells would be activated by antigen before the expression of anti-Id-induced suppressor T cell activity allowing them to escape suppression.

It was also demonstrated that Id^+ T cells can exert suppressor activity in the presence of antigen alone. Removal of Id^+ cells on anti-Id-coated plates resulted in the enhancement of T cell proliferation and helper function. Suppression of immune responses by antigen-binding $Lyt\text{-}2^+$ T cells has

TABLE II
INDUCTION OF SUPPRESSOR T CELLS BY ANTIIDIOTYPIC ANTIBODY[a]

Subject number	Preincubation of T cells added to the culture	Proliferation of culture stimulated with		
		—	TT	DT
1	Medium	187 ± 63[b]	14,556 ± 1,280	13,782 ± 583
	Rabbit preimmune IgG	196 ± 28	13,533 ± 622	13,805 ± 497
	Rabbit anti-Id IgG	265 ± 41	*8,677 ± 447*	13,725 ± 721
2	Medium	585 ± 36	17,503 ± 1,105	8,615 ± 263
	Rabbit preimmune IgG	643 ± 28	16,825 ± 648	9,376 ± 408
	Rabbit anti-Id IgG	609 ± 64	*9,672 ± 579*	8,861 ± 225
3	Medium	428 ± 45	12,392 ± 272	10,706 ± 320
	Rabbit preimmune IgG	531 ± 43	12,014 ± 380	9,814 ± 286
	Rabbit anti-Id IgG	516 ± 29	*7,631 ± 549*	9,356 ± 351

[a] T cells were preincubated for 2 days with rabbit IgG (250 μg/ml), were washed, and were added at a 1:1 ratio to untreated, autologous T cells in the presence of 20% autologous monocytes. Results represent mean ± SE of triplicate cultures. Values in italics were significantly different ($p < 0.05$) from controls.

[b] Counts per minute of [^{3}H]thymidine incorporated per culture.

TABLE III
INDUCTION OF SUPPRESSOR T CELLS BY ANTIIDIOTYPIC ACTIVITY[a]

Donor number	Preincubation of T cells added to the culture	IgG anti-TT (ng/ml) in TT-stimulated cultures	IgG anti-DT (ng/ml) in DT-stimulated cultures
1	Medium	126 ± 9	84 ± 7
	Rabbit preimmune IgG	115 ± 12	81 ± 11
	Rabbit anti-Id IgG	*78 ± 6*	80 ± 8
2	Medium	89 ± 8	67 ± 14
	Rabbit preimmune IgG	93 ± 10	65 ± 8
	Rabbit anti-Id IgG	*56 ± 4*	62 ± 9
3	Medium	95 ± 11	77 ± 5
	Rabbit preimmune IgG	88 ± 6	81 ± 12
	Rabbit anti-Id IgG	*53 ± 7*	75 ± 8

[a] Results represent mean ± SE of triplicate cultures. T cells were preincubated for 2 days with rabbit IgG (250 μg/ml), were washed, and added to autologous T and B cells at a ratio of 2:2:1 and the cultures were stimulated 4 days with antigen (TT and DT, 20 ml), washed, and recultured for 8 days. Supernatants were then collected and assayed for IgG anti-TT and IgG anti-DT. Cultures not stimulated with antigen made neither IgG anti-TT nor IgG anti-DT. IgG anti-TT was present only in TT-primed cultures and IgG anti-DT was present only in DT-primed cultures. Values in italics were significantly different ($p < 0.05$) from controls.

been described in experimental animals (Tables II and III) (Taniguchi and Miller, 1977).

D. THE AUTO-ANTI-Id ANTIBODIES IN THE NORMAL HUMAN IMMUNE RESPONSE

Auto-anti-Id to anti-TT antibodies was demonstrated in the circulation of normal adults following booster immunization with TT. IgG (Geha, 1982a) capable of binding autologous IgG $F(ab')_2$ anti-TT, derived from serum obtained 7 and 10 days after immunization, was detectable in the serum by the end of the second week postimmunization (Fig. 6). This binding was shown to be idiotype specific and not to result from rheumatoid factor-like activity. Auto-anti-Id antibodies detected at different intervals postimmunization recognized overlapping but also different idiotypes on IgG $F(ab')_2$ anti-TT obtained shortly postimmunization (days 7–10).

Auto-anti-Id IgG was shown to recognize idiotypic determinants involved in TT binding because IgG taken 8 weeks and more after immunization and depleted of anti-TT reactivity specifically inhibited the binding of ^{125}I-labeled TT to autologous IgG collected at days 7 and 10 postimmunization. Significant auto-anti-Id activity was still detectable in both assays (idiotype binding and inhibition of TT antigen binding) at the end of the study period 4

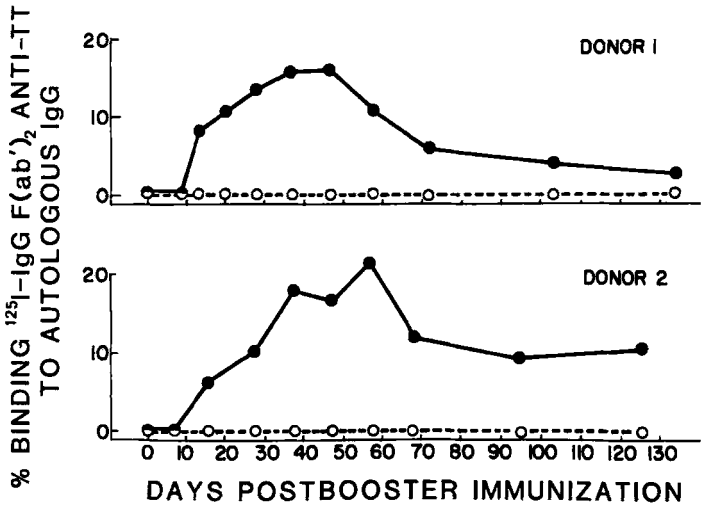

FIG. 6. Binding of ^{125}I-labeled IgG F(ab')$_2$ anti-TT to autologous IgG as determined by precipitation with *S. aureus*. Numbers on the abscissa represent the days postbooster immunization with TT. The numbers on the ordinate represent percentage binding of 20 ng (50,000 cpm) of ^{125}I-labeled IgG F(ab')$_2$ anti-TT (●) and of 20 ng of ^{125}I-labeled TT-nonreactive IgG F(ab')$_2$ (○) to 250 μg of autologous IgG. Percentage binding was calculated by using as total binding the amount of IgG F(ab')$_2$ bound by rabbit anti-human Fab. In the case of IgG F(ab')$_2$ anti-TT, the amount bound to Sepharose 4B TT was almost (>90%) equivalent to the total binding. The cpm of ^{125}I-labeled IgG F(ab')$_2$ anti-TT bound to *S. aureus* alone was 190 cpm in donor 1 and 85 cpm in donor 2. All tests were done in triplicate and repeated twice. (From *J. Immunol.* **129**, 141, 1982.)

months postimmunization. In experimental animals the duration of the auto-anti-Id response has been reported to vary in length from 2 weeks to a few months (8 to 15). This variability probably results from difference in antigens used for immunization, modes of immunization, and sensitivity of the assays used to detect auto-anti-Id antibody. Our findings of a prolonged auto-anti-Id response after booster immunization with TT may be due to a long life of anti-Id antibody-secreting B cells and/or to continuous stimulation of anti-Id synthesis by free antibody and/or by idiotype-bearing cells.

To study the modulation of the expression of idiotypes by circulating IgG after immunization with TT, a rabbit anti-Id antibody was used as a probe. This rabbit anti-Id was raised against IgG F(ab')$_2$ anti-TT present at days 7 and 10 postimmunization, i.e., against "early" anti-Tt idiotypes. The antiserum precipitated only 35 to 40% of the immunizing IgG F(ab')$_2$ anti-TT. The reactivity of this anti-Id with IgG diminished starting 2 weeks postimmunization and bottomed at about 2 months postimmunization at a time of peak auto-anti-Id activity (Fig. 7) indicating a decrease in the level of "early"

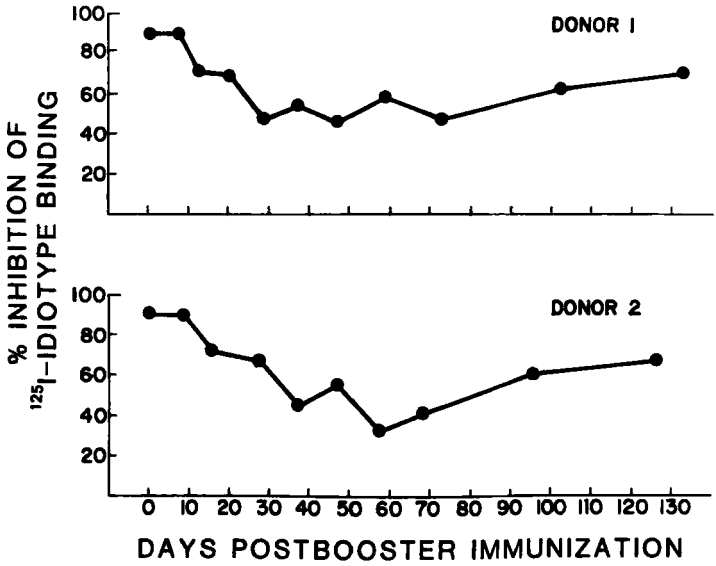

FIG. 7. Inhibition of the binding of ^{125}I-labeled "idiotype" [IgG F(ab')$_2$ anti-TT] to rabbit antiidiotypic IgG derived from the idiotype donor. In these experiments 500 μg of rabbit anti-Id IgG and 20 ng (3000 to 5000 cpm/ng) of ^{125}I-labeled idiotype were used. IgG from the idiotype donor was used in the amount of 250 μg. (From *J. Immunol.* **129**, 143, 1982.)

anti-TT idiotypes. The decrease in the expression of "early" anti-TT idiotypes was long lasting, for it remained evident 4 months after booster immunization, a time by which the levels of auto-anti-Id were greatly reduced. Although the mechanisms responsible for this long-lasting modulation of idiotype expression remains unknown, it is clear that the decrease in the expression of "early" anti-TT idiotypes took place concomitantly with the appearance of auto-anti-Id antibody. These results and those of Schrater and co-workers (1979) in the mouse suggest that the auto-anti-Id response may have contributed to the modulation of idiotype expression observed after booster immunization with TT.

The modulation of idiotype expression after immunization with TT must not have been limited to a decrease in the levels of some of the "early" anti-TT idiotypes, because this decrease occurred, in the face of a rise in hemagglutinating IgG antibody titers. Thus, it is quite probable that there was a concomitant increase in the levels of other anti-TT idiotypes. Similar conclusions were reached previously by MacDonald and Nisonoff (1970) in their longitudinal study of idiotype expression in rabbits repeatedly immunized with the *p*-azobenzoate hapten.

The presence of circulating auto-anti-Id was associated with the presence

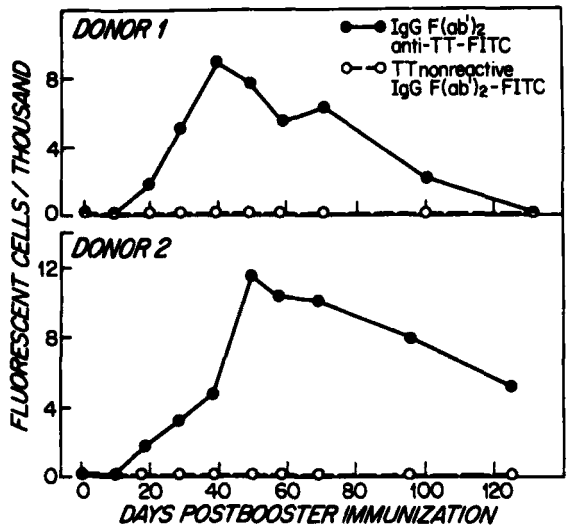

FIG. 8. Binding of nonadherent peripheral blood lymphocytes to fluorescein-conjugated autologous IgG $F(ab')_2$. Numbers on the abscissa represent days after booster immunization with TT. Numbers on the ordinate represent the frequency of cells staining with autologous IgG $F(ab')_2$ anti-TT (●) and autologous TT-nonreactive IgG $F(ab')_2$ (○). (From *J. Immunol.* **130**, 1634, 1982).

of circulating Id binding cells. Indeed circulating cells capable of binding autologous IgG $F(ab')_2$ anti-TT appeared by the third week after boosting and remained detectable throughout the 3- to 4-month study period (Fig. 8). The frequency of idiotype binding cells paralleled more or less the magnitude of the serum auto-anti-Id antibody response, which suggests that auto-anti-Id bearing cells can remain activated for prolonged periods of time after immunization.

Because of the presence of auto-anti-Id antibody in the serum, great care was taken to ascertain that the binding of autologous IgG $F(ab')_2$ anti-TT to peripheral blood lymphocytes (PBL) was not due to passively absorbed auto-anti-Id. Since idiotype binding cells could be detected after treatment of the PBL with trypsin and after overnight incubation to allow for the shedding of passively absorbed immunoglobulins, it is safe to conclude that passively absorbed auto-anti-Id did not play a significant role in the experiments. This was further confirmed by the demonstration that pokeweed mitogen could induce the synthesis of auto-anti-Id *in vitro*.

The idiotype binding cells detected were predominantly B cells. Very few idiotype binding T cells were detected. This, however, should not be interpreted to indicate absence of auto-anti-Id-bearing T cells. In experimental animals several investigators have presented evidence for auto-anti-Id-

bearing T cells. Tasiaux *et al.* (1978) have detected by immunofluorescent techniques auto-anti-Id-bearing cells in rabbits immunized repeatedly with tobacco mosaic virus. Janeway *et al.* (1980) and Woodland and Cantor (1978) have presented functional evidence for a T helper cell that recognizes idiotypes expressed on B cells, and Ju *et al.* (1976) and Weinberger *et al.* (1980) have presented evidence for antiidiotype-bearing suppressor effector T cells.

A direct affect of auto-anti-Id on TT antigen-specific antibody synthesis could be demonstrated. Auto-anti-Id (day 38 IgG, Fig. 6) partially but significantly inhibited the PWM-induced and the spontaneous synthesis of anti-TT antibody by PBL taken shortly (10 days) after immunization (Fig. 9). The inhibition of anti-TT synthesis by auto-anti-Id was antigen specific and idiotype specific. Since auto-anti-Id antibody recognizes only a fraction of the idiotypes expressed by IgG anti-TT and presumably only a fraction of the anti-TT idiotypes expressed by PBL taken shortly after immunization, its effect on total IgG anti-TT synthesis was only a partial effect, albeit a significant one. It was directly demonstrated that the synthesis of those idiotypes that were recognized by the auto-anti-Id antibody was the one predominantly affected. Anti-TT synthesis by PBL present in the circulation at the same time as the auto-anti-Id antibody was not affected by that antibody (Fig. 9), which suggests that some anti-TT idiotypes expressed by PBL early

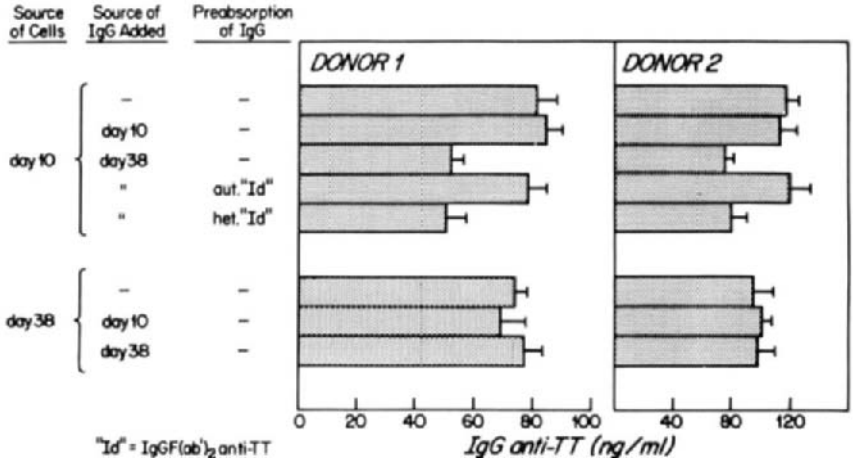

FIG. 9. Effect of IgG taken postimmunization on IgG anti-TT synthesis by PWM-stimulated autologous PBL. Taken 10 days after and 38 days after booster immunization with TT in the two subjects studied. Number of days represents days elapsed after booster immunization. Values represent mean ± SD of triplicate cultures. IgG samples tested for their capacity to inhibit IgG anti-TT synthesis were preabsorbed extensively with Sepharose 4B-TT. Absorptions with "Id," i.e., IgG $F(ab')_2$ anti-TT, were performed by absorbing 2.5 mg of IgG in 1 ml of PBS with 0.25 mg of IgG $F(ab')_2$ anti-TT cross-linked to 0.25 ml of Sepharose 4B. (From *J. Immunol.* **130**, 1634, 1982.)

after immunization are no longer expressed later in the course of the immune response.

In contrast to TT antigen, which stimulates anti-TT synthesis at low doses (Volkman *et al.*, 1981), auto-anti-Id failed to stimulate anti-TT synthesis when added at low concentrations to cultures of PBL taken at day 0 or day 10 postimmunization. This may have resulted from the failure of auto-anti-Id antibody to activate TT-specific helper T cells and/or because of selective activation of suppressor T cells. In this regard there is evidence that the TT determinants recognized by helper T cells differ from those recognized by serum antibody to TT (Broff *et al.*, 1981). Although anti-Id antibody cross-reacts with TT determinants recognized by antibody, it is likely that it does not cross-react with those determinants recognized by the helper T cells and hence fails to activate these T cells.

E. Naturally Occurring Auto-Anti-Id Antibodies

Naturally occurring antibodies to human immunoglobulin $F(ab')_2$ fragments have been described in the IgM fraction of mixed cryoglobulins (Geltner *et al.*, 1980) as well as in normal serum (Bankert and Pressman, 1976; Nasu *et al.*, 1980). In addition Cunningham-Rundles (1981) has documented the presence of anticasein antibodies in the sera of otherwise healthy IgA-deficient subjects, Bose *et al.* (1984) have reported auto-anti-Id antibodies to grass-specific antibody in patients receiving immunotherapy with grass, and Saxon and Barnett (1984) have reported auto-anti-Id antibodies to TT-specific T cells in the sera of subjects extensively hyperimmunized with TT. None of these studies examined whether the detected anti-Id were capable of modulating the antigen-specific immune response. The finding of human auto-anti-Id antibodies in disparate situations, together with the established role in modulating the immune response in animals and in the case of anti-TT response in man, make it likely that these antibodies contribute to immunoregulation in man.

F. Auto-Anti-Id Antibody in Abnormal Human Immune Responses

The relatively frequent presence of serum immunoglobulin M components in patients with immunodeficiency raises the question as to whether a deficient idiotype network in such patients results in failure to self-regulate immunoglobulin production by anti-Id. In one such patient who was extensively studied and who was suffering from hypogammaglobulinemia the majority of circulating IgG was accounted for by an $IgG_{1,\kappa}$ M component. *In vitro* synthesis of this M component could be profoundly inhibited by anti-idiotypic IgG raised in a rabbit (Mudawwar *et al.*, 1980). It is tempting to speculate that failure to make an effective regulatory auto-anti-Id response in

this immunodeficient patient set the stage for the unregulated synthesis of the M component. Such a hypothesis may not only be applicable to patients with generalized immunodeficiency and M component but perhaps also to patients with B cell proliferation with or without M component. Heterologous anti-Id has been shown to inhibit B cell production of M components *in vitro* in some of these patients.

V. Idiotypic Interactions and the Allergic Response

In animals IgE antibodies have been shown to share idiotypic determinants with antibodies of the IgG isotype. Furthermore anti-Id antibodies have been shown to down-regulate the IgE antibody response in experimental animals (Blaser *et al.*, 1980; Dessein *et al.*, 1980).

Sharing of idiotypic determinants between IgE antibodies and IgG antibodies has also been shown in humans (Geha, 1981). Indeed, rabbit anti-Id antibodies to human IgG anti-TT elicited a Prausnitz–Kustner reaction in sites of the forearms of normal subjects sensitized with IgE anti-TT antibodies derived from the same donor of the IgG anti-TT antibodies used for immunization. It was very important in these studies to ascertain that the material used to raised the anti-Id consisted solely of IgG anti-TT and was not contaminated with IgE anti-TT. This was done by absorbing the immunosorbent purified IgG $F(ab')_2$ anti-TT with Sepharose-anti-IgE prior to its injection into the rabbit. The resultant injected material contained no detectable IgE. Thus the elicitation of a wheal and flare reaction by the anti-Id in skin sites sensitized with serum from the "idiotype" donor was indeed due to shared Id determinants between IgG and IgE.

As in the case of IgG, anti-Id antibodies were demonstrated to inhibit the antigen-specific IgE antibody response *in vitro* (Geha and Comunale, 1983). Lymphocytes from donors whose serum contained IgE antibodies to TT synthesized IgE anti-TT *in vitro* spontaneously following immunization. This synthesis was inhibited upon addition of anti-Id antibodies. The target of anti-Id suppression appeared to be B cells as well as radio-sensitive T cells. Indeed anti-Id inhibited IgE anti-TT synthesis by cultures of purified B cells. Furthermore, T cells preincubated with anti-Id then washed, inhibited IgE anti-TT synthesis by B cell cultures.

Repeated immunization with antigen appears to be conducive to the production of auto-anti-Id as previously discussed. Perhaps this is because complexes of antigen and Id are particularly immunogenic. Allergic diseases involve repeated stimulation by antigen either naturally or in the course of immunotherapy. Thus the presence of auto-anti-Id antibodies to allergen-specific antibodies in humans would be expected. Auto-anti-Id has been described in isolated allergic patients receiving immunotherapy (Bose *et al.*,

1984). In a rather large series Castracane *et al.* (1985) have reported the presence of auto-anti-Id to ragweed antibodies in normal subjects ($n=12$) and to a lesser extent in untreated allergic subjects ($n=13$). In allergic subjects treated with immunotherapy ($n=7$) the level of auto-anti-Id appeared to go up to the normal range. These results suggested that decreased production of auto-anti-Id in allergic subjects allows the escape of IgE antibody synthesis from immunoregulatory influences.

Allergen-specific antibodies have been in general found to share cross-reactive idiotypes CRI (Bose *et al.*, 1984; Castracane *et al.*, 1985). These CRI may be the result of a limited number of V genes committed to the antibody response to allergens which are proteins of rather low molecular weight. CRI may be further heightened by repeated antigenic stimulation which in animals leads to the emergence of dominant idiotypes (Conger *et al.*, 1981). It must be noted, however, the presence of anti-Id antibodies bearing internal image of antigen in anti-Id reagents and/or poor absorption of antibodies directed against a nonhypervariable region of the V domain represented in a small proportion of human immunoglobulins (Kunkel, 1984) may have contributed to the high degree of idiotypic cross-reactivity observed in some studies.

Taken together the data discussed strongly suggest a role for auto-anti-Id antibody in the regulation of the allergic response. Longitudinal studies that examine the level of auto-anti-Id, the modulation of idiotypes on allergen specific IgE, and the effect of auto-anti-Id on IgE synthesis still need to be performed.

VI. Idiotypic Interactions in Self-Tolerance and Autoimmunity

A. The Idiotypic Network and Self-Tolerance

Rather than invoking a variety of different mechanisms, e.g., clonal abortion, suppressor T cells, tolerance induction, it has been suggested that self–non-self discrimination is determined by the idiotype network. The assumption is that genes of the immune system have evolved so as to encode antibodies that recognize self-antigens. If DNA rearrangements of mRNA processing were designed so as to make a sufficient number of mistakes, then the set of antibodies that these genes would fail to express would be precisely the anti-self antibodies which they encode. However, faithful expression of the anti-self encoding genes does occur to some extent, resulting in the production of anti-self Ig molecules. But these, binding to self, then invoke the production of antiidiotypic molecules (Cleveland *et al.*, 1983). Antiidiotypic antibodies can prevent, as experiments have shown, the future

expression of the corresponding idiotypes (Cosenza and Kohler, 1982). The idea that antiidiotypic suppression helps the immune system to acquire self-tolerance should be linked with the idea of idiotype–antiidiotype "partners" in the idiotypic network discussed above. If normal immunoglobulin is a mainly stable population of molecules that are antiidiotypic to the anti-self molecules likewise encoded in the germ line as suggested by the studies of Holmberg *et al.* (1984), thus the most frequent rearrangements of *V*, *D*, and *J* gene segments, and the most frequent heavy and light chain partners, determine the structures of both anti-self molecules and of corresponding antiidiotypic molecules. The latter, in response to self–anti-self complexes, remain in the majority and exercise idiotypic suppression. A person who has blood group A makes antiidiotypic antibodies only to anti-A antibodies, whereas the anti-B antibodies do not invoke sufficient concentrations of suppressive antiidiotypic antibodies. A small fraction of B cells that produce anti-self molecules may escape idiotypic suppression. Even anti-self antibodies that are known to cause autoimmune diseases, when occurring in large concentrations, have been shown to be present at low levels in normal healthy subjects (Witebsky *et al.*, 1957). In both human and mouse, antibodies to self-antigens, e.g., DNA epitopes and to immunoglobulin (Ig) epitopes and idiotopes have been reported to display a high incidence of cross-reactive idiotypes (CRI) (Kunkel *et al.*, 1973; Matsuyama *et al.*, 1983; Solomon *et al.*, 1983; Feizi *et al.*, 1974; Vincent, 1981). This could reflect the presence of conserved germ line *V* regions that encode variable regions that bind to the limited immunogenic epitopes on self antigens.

B. The Idiotypic Network and Autoimmunity

The regulation of the production of anti-DNA antibodies by anti-Id antibodies has been studied in systemic lupus erythematosus (SLE) (Abdou *et al.*, 1981a). Various sera from SLE patients in active and inactive states of their disease, as well as sera from normal individuals, were first completely depleted of anti-DNA and of DNA by affinity chromatography. The suppressive capacity of equimolar concentrations of the various depleted sera (blocking sera) on target lupus sera were determined. The target sera were from lupus patients with known DNA-binding capacity. Blocking sera from inactive SLE suppressed the binding of autologous anti-DNA antibody to [^{3}H]DNA. Blocking sera from active SLE, as well as human serum albumin, did not suppress. Sera from normal donors who had no contact with lupus patients or with lupus sera did not suppress, whereas those from normal donors who had contact with lupus patients or sera did suppress the binding. The anti-anti-DNA antibody suppressive activity in the inactive lupus serum was shown to be localized within the $F(ab')_2$ portion of immunoglobulin by normal human γ-globulin. Furthermore, immune complexes could be de-

tected by a C1q binding assay when the inactive lupus blocking sera were incubated with the anti-DNA antibody containing target sera. These findings suggested regulation of serum anti-DNA antibody levels by anti-Id antibodies could induce and maintain disease remission in lupus patients and prevent disease expression in normals. These auto-anti-Id to DNA were shown to regulate anti DNA synthesis *in vitro* (Abdou *et al.*, 1981a). $F(ab')_2$ fragments were found to bind to autologous T cells, to allogeneic lupus T cells, but not to normal T cells. T cells from patients with inactive lupus incubated with anti-Id but not T cells of active SLE patients suppressed anti-DNA secretion but not polyclonal IgG secretion by autologous cells. This suggested that anti-Id in inactive SLE activates clonal suppressor T cells that regulate the specific autoantibody.

C. Antiidiotype and Regulation of the Antibody Response Which Cross-Reacts with Self

In one model of autoimmune diseases a foreign agent (virus, bacteria, protein, toxin) enters the body. An antibody response is mounted (Ab1). If the agent shares an antigenic site with a body constituent (e.g., hormone, cell surface structure, receptor site) Ab1 will cross-react with this constituent. This may lead to tissue destruction and/or interfere with function.

It has been shown that the titer of autoantibodies is often increased both during and after viral infections (Fong *et al.*, 1981; Kurki *et al.*, 1978; Toh *et al.*, 1979; Haire, 1972). Herpes simplex virus and measles virus, for example, contain proteins that share antigenic determinants with human intermediate filaments (Fujinami *et al.*, 1983). The sharing of antigenic determinants by proteins in neurons affected by Chaga's disease and by proteins of *Typanosoma cruzi* (Wood *et al.*, 1982) is an example of how a parasitic infection may be responsible for an autoimmune process. The demonstration that animals immunized with group A *Streptococcus*, develop antibodies that react with myocardium (van de Rijn *et al.*, 1977) suggests the potential association between rheumatic heart disease and streptococcal infections. In addition murine monoclonal antibodies to *Streptococcus pyogenes* reacted with skeletal muscle myosin (Krisher and Cunningham, 1985). High-molecular-weight proteins in extracts of human heart tissue that reacted with an antibody to S. *pyogenes* also reacted with a monoclonal antibody to ventricular myosin. Adsorption of the antibody to streptococci with S. *pyogenes* simultaneously removes reactivity of the antibody for either S. *pyogenes* or myosin. Thus myosin shares immunodeterminants with a component of S. *pyogenes*.

The α-subunit of the acetylcholine receptor and two membrane proteins from *E. coli*, as well as two protein from *K. pneumoniae*, share at least one antigenic determinant, and the α-subunit of the acetylcholine receptor and a

protein in *Proteus vulgaris* also share at least two antigenic determinants (Stefansson *et al.*, 1985).

E. coli has been shown to contain material reactive with antibodies against insulin and brain gangliosides (LeRoith *et al.*, 1981; Soderstrom *et al.*, 1984). *S. epidermidis, E. coli,* and *Pseudomonas maltophilia* have substances that react with antibodies against chorionic gonadotropin (Maruo *et al.*, 1979), and some unspecified glycopeptides from human and rat brains have also been shown to share antigenic determinants with meningococcus group B and *E. coli* K1 (Finne *et al.*, 1983). In addition, several lines of evidence indicate that plasma membranes from human thyroid and *Yersinia enterocolitica* share antigenic determinants (Weiss *et al.*, 1983).

The fact that foreign proteins and self share antigenic determinants does not explain why antibodies to self develop in a certain patient, nor does it explain why all persons do not have autoimmune disease. One mechanism by which the production of antiself antibodies is allowed would be a factor of antiidiotypic regulation as discussed in the previous section.

D. Anti-Id Antibody as Mediators of Autoimmune Disease

In yet another model of autoimmune disease antibodies to a foreign agent (Ab1) elicit the formation of anti-Id antibodies (Ab2). Some of these anti-Id antibodies may represent the internal image of antigen and hence can bind to the same sites as the foreign agent thus perpetuating tissue damage. If the membrane site is also a hormone receptor site the antiidiotypic antibody (Ab2) will appear to be a receptor antibody and may mimic the action or block the action of the hormone (Fig. 10).

Anti-Id antibodies to antibodies directed against β-adrenergic agents and insulin have agonistic effects (Guillet *et al.*, 1985; Schechter *et al.*, 1982). The presence of anti-B2 receptor antibody in patients with severe asthma may represent yet another instance of anti-Id antibody bearing the internal image of antigen. Anti-Id to phosphorylcholine antibodies cross-reacts with acetylcholine esterase inhibitor (Strickland *et al.*, 1985). In the most compelling example, an autoimmune illness was caused by antiidiotype antibodies. Antibodies were raised to an acetylcholine analog, and were shown to resemble the acetylcholine receptor in fine detail with respect to the binding of acetylcholine analogs (Wasserman *et al.*, 1982; Cleveland *et al.*, 1983). Rabbits immunized with this receptor-mimicking antibody not only responded with antiidiotype antibodies, but also began to have a myasthenia gravis-like illness resembling that produced by anti-acetylcholine receptor antibody. The presence of anti-Id antibodies to CRI on anti-acetylcholine antibodies in patients with myasthenia gravis (Vincent, 1981) may contribute to the presence of antireceptor antibodies in these patients. In a similar vein an experi-

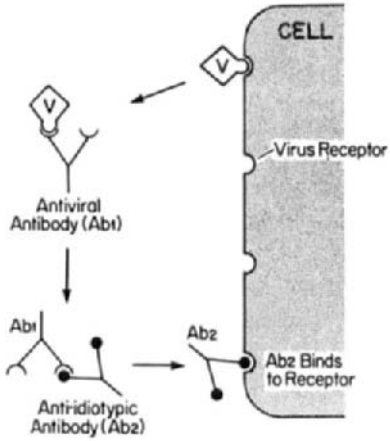

FIG. 10. Antiidiotype antibody (Ab2) against an antibody to a virus (Ab1) that binds to a cell surface may act as an antibody against the cell surface receptor.

mental model of Graves' disease has been produced in rabbits immunized with TSH and making auto-anti-Id antibodies (Beale *et al.*, 1985).

Finally it is intriguing that the antigens to which many autoantibodies are directed in several apparently autoimmune illnesses have a striking similarity: all are structures which viruses are likely to depend upon in a critical way for nucleic acid reproduction, protein synthesis, or genome insertion, e.g., anti-RNP antibodies are directed at an RNP–protein complex whose function is to recognize the 5′-splicing site of a premessenger RNA.

VII. Idiotypic Network Regulations of Immune Responses to HLA Antigens

As long as a decade ago specific transplantation tolerance was induced in experimental animals by immunization with alloantigens (McKearn *et al.*, 1974) or by autoimmunization against the individual's own naturally occurring idiotype antigen binding receptors (Binz and Wigzell, 1976). The auto-anti-Id antibodies and auto-antiidiotypic T cells induced against T cells alloreactive to specific transplantation antigen act to suppress the recipients' response *in vivo* to the transplantation antigens leading to organ graft survival.

In recent studies involving bone marrow transplantation from parent to F_1 in rats auto-antiidiotypic cytotoxic T cells were shown to play a role in preventing graft-versus-host disease (Kimura and Wilson, 1984). Transfer of parental strain T lymphocytes into F_1 hybrid rats differing with respect to gene products of the major histocompatibility complex (MHC) causes a graft-versus-host (GvH) reaction. This reaction results from recognition of host allogeneic MHC gene products by specific clones of donor T cells. When

given systemically in sufficient numbers, these donor T cells cause a progressive, generally fatal wasting syndrome. A more local, nonfatal GvH reaction, marked by extensive enlargement of the draining lymph nodes, ensues when donor T cells are administered via the footpad. Cells derived from the enlarged draining lymph nodes of (A × B) F_1 animals undergoing local GvH disease caused by donor A T cells were shown to contain a subpopulation of host derived killer T cell precursors which can be activated to lyse specific blast cells, derived from mixed lymphocyte culture (MLC), reactive to host MHC alloantigens. These "antiidiotypic" cytolytic T cells lyse A anti-MHC MLC blasts, and also, they lyse A anti-MHC MLC blasts from MHC different, third party rat strains. The latter finding suggests the presence of cross-reactive idiotypes on T cell receptors for alloantigen.

The applicability of the above findings in experimental animals to humans is suggested by the finding that T cells alloactivated in 5-day MLC mixed lymphocytes with an HLA-DR different stimulator acquire the capacity of stimulating the autologous mixed lymphocyte response (AMLR) (Suciu-Foca *et al.*, 1982). Activation of AMLR by allosensitized T cells is determined by the expression of the idiotype receptor for the stimulating HLA-DR alloantigen. This has been shown in experiments in which purified, OKT3-positive T cell suspensions were first primed for 9 days with AMLR-activated T lymphoblasts, then tested in secondary AMLR with autologous lymphoblasts sensitized to various HLA-DR alloantigens. Accelerated memory responses were induced only by autologous primary AMLR stimulators. This response was not inhibited by a mouse monoclonal antibody recognizing Ia-like determinants and was not triggered by human allogeneic resting peripheral blood lymphocytes. Thus, recognition of alloactivated T lymphoblasts in secondary AMLR seems to be specific for the idiotype-like determinants expressed by the autologous stimulators.

In humans, alloimmunization through pregnancy, blood transfusion, or organ transplantation leads to the development of antibodies directed against HLA antigens. Surprisingly, anti-HLA antibodies are found only in approximately a quarter of multiparous women, although they all had been exposed to the fetus' paternal HLA antigens which pass the placental barrier during pregnancy. Furthermore, kidney transplants from children to their mothers or fathers survive at the same rates, suggesting that mothers are not immunized to their offspring. Also among dialysis patients transfused repeatedly with blood from a single donor only 30% develop anti-HLA antibodies. Interestingly, transplantation of a kidney from the actual blood donor into a patient who did not form anti-HLA antibodies results in the highest survival rates.

Studies by Reed *et al.* (1983) and Suciu Foca *et al.* (1983a,b) suggest that tolerance of foreign HLA antigens may be mediated by anti-Id antibodies directed against anti-HLA antibodies as well as against alloresponsive T

cells. Antiidiotypic antibodies were detected in the serum of multiparous women. These antibodies inhibited the reactivity of autologous antibodies to the DR antigens of the husband. The reactivity of sera from other individuals to the husband's DR antigens was inhibited suggesting the presence of cross-reactive idiotypes among antibodies to HLA determinants and that germ line encoded regulatory idiotypes seem to be involved in anti-HLA immunization. Furthermore it was shown that sera from parous women react with autologous T lymphoblasts primed in 5-day mixed lymphocyte culture against their husband, i.e., with lymphoblasts expressing receptors for the immunizing donor. Anti-HLA receptors expressed by T and B lymphocytes appeared to share serologic determinants because sera that bind to autologous alloactivated lymphoblasts were also capable of inhibiting the anti-HLA activity of autologous and homologous sera. Direct binding of maternal IgG $F(ab')_2$ to autologous alloactivated T cells responding to the husband's lymphocytes was shown. Furthermore maternal $F(ab')_2$ triggered the proliferation of these autologous blasts but not of autologous blasts primed against a third party.

To document the relevance of antiidiotypic immunity to HLA in pregnancy, the relationship of Ab1 to Ab2 before and after delivery was investigated (Suciu-Foca *et al.*, 1983a). Ab1 was assessed by the capacity of sequential samples of serum obtained before and after delivery to bind to the husband's lymphocytes. The titer of the Ab1 was determined by serial dilutions. After absorption of Ab1 on lymphocytes from the immunizing donor, the Ab2 activity of each of the sequential samples was determined by its capacity to block binding of nonabsorbed autologous serum to the father's lymphocytes and to an HLA reference panel. The titer of Ab2 was inferred from the efficiency of blocking serial dilutions of Ab1. The efficiency of blocking was highest during pregnancy, i.e., there was an inverse correlation between the titer of Ab1 and Ab2 with the latter showing the highest levels during pregnancy and at the time when Ab1 disappears. At delivery, a high titer of Ab1 was found while Ab2 had decreased significantly. Postpartum, with the decrease of Ab1, the titer of Ab2 rose again. The prevalence of AB2 during pregnancy, followed by a temporary decline postpartum at the time when Ab1 increases, and by a rise with the decrease in titer of Ab1, strongly suggests that antiidiotypic antibodies to HLA play an important role in down-regulating maternal immunity to the fetus.

Because auto-antiidiotypic antibodies were found in sera from all parous women tested, the hypothesis that nonresponsiveness to alloantigens exists as a state per se is not likely. The passive transfer of antireceptor (idiotype) immunity by use of antibodies from pregnant women's sera may provide a powerful tool for specific suppression of allograft rejection.

Mechanisms involving antiidiotypic antibodies similar to those presumed to operate in the tolerance of fetal allograft may be operative in the tolerance

to organ grafts. Thus auto-anti-Id antibody to anti-HLA antibodies and anti-HLA-DR cell receptors may explain the success of kidney grafts in multiply transfused individuals.

VIII. Antiidiotypic Antibodies as Vaccines

The use of antiidiotypes as vaccines is based on the observation that administration of anti-Id can induce the production of antigen binding positive molecules in the absence of exposure to antigen. These molecules arise because they are complementary to the anti-Id. As documented in many systems idiotype-positive non-antigen binding molecules may also arise (Oudin and Cazenvae, 1971; Eichmann *et al.*, 1977; Wysocki and Sato, 1981). It is also possible if the injected anti-Id bears the internal image of antigen to give rise to antigen binding antibody molecules some of which may not bear the complementary idiotypes.

The applicability of anti-Id as vaccines has been demonstrated in several studies involving the induction of protective immunity against bacterial, viral, and parasitic antigens. McNamara *et al.* (1984) used a monoclonal antiidiotype antibody specific to the binding site of the anti-phosphorylcholine myeloma TEPC-15 to immunize BALB/c mice against a lethal *Streptococcus pneumoniae* infection. Vaccinated mice developed a high titer of antibody to phosphorylcholine, which is known to protect against infection with S. *pneumoniae.* Measurement of the median lethal dose of the bacteria indicated that antiidiotype immunization significantly increased the resistance of BALB/c mice to the bacteria challenge.

Kennedy and Dressman (1984) used rabbit antiidiotype reagents directed against an idiotype shared by human and murine antibodies to hepatitis B surface antigen. Prior injection of antibodies to the idiotype into mice markedly increased the number of spleen cells secreting immunoglobulin M (IgM) anti-HBs and increased serum anti-HB-S titers when the mice were subsequently inoculated with HBsAg. Anti-HBs-secreting cells could be induced solely by injecting idiotype antibodies. This anti-HBs expressed the interspecies idiotype and recognized an immunogeneic determinant common to the four major serotypes of HB conferring protection.

Uytdehaag and Osterhaus (1985) raised a syngeneic monoclonal antiidiotypic antibody against an idiotope on a protective monoclonal antibody with specificity for poliovirus type II. The anti-Id detected an interspecies cross-reactive idiotope. Injection of the anti-Id into mice induced antibodies of complementary specificities. These antibodies were antigen binding and neutralized poliovirus II *in vitro.* However the *in vivo* the neutralizing antibody titer was not sufficient to protect against infection of the immunized mice with poliovirus.

Protective immunizations of experimental animals against two of the most

common parasitic infections which affect man and which so far have resisted attempts at control, namely trypanosomiasis and schistosomiasis, have been achieved by injection of anti-Id antibodies. Sacks *et al.* (1982) injected mice with anti-Id antibodies to monoclonal antibodies protective against *Trypanosoma rhodesiense*. Immunity against homologous challenge ensued as manifested by either complete protection or reduced parasitemia. There was a rapid appearance and achievement of higher levels of the idiotypes in treated animals compared to untreated animals following their infection. Recently Grzych *et al.* (1985) studied the capacity of immunization with anti-Id antibody to protect rats against schistosomal infection. They raised anti-Id antibodies to a monoclonal antischistosomal antibody IPLSm1 which was known to exhibit marked cytotoxicity for schistosomula in the presence of eosinophils and a high degree of protection by passive transfer. This antibody was directed to a highly glycosylated 38 kDa protein. This protein by itself induced blocking antibodies which inhibited the properties of IPLSm1 and thus the proteins could not be used as a protective vaccine. In contrast injection of anti-Id (Ab2) into rats resulted in the production of anti-schistosomal-specific antibodies which bound to the 38kDa protein and which, most importantly, were cytotoxic to schistomula in the presence of rat eosinophils *in vitro*. Animals producing these antibodies demonstrated marked protection to a challenge infection. This protection could be passively transferred to other rats by serum from the anti-Id immunized rats.

Taken together the above observations suggest that anti-Id can be used successfully as vaccines. For general applicability the following conditions must be met for anti-Id to be used as vaccines. (1) The anti-Id must be directed against protective antibodies. (2) The protective idiotypic antibodies must bear cross-reactive idiotypes. (3) The antigen to be bypassed in the immunization procedure must be difficult to produce, unsafe, or poorly immunogenic. Given the above set of conditions anti-Id antibodies could be used in humans. They could be directed against human antibodies obtained from serum or from B–B hybridoma which produce protective antibodies bearing cross-reactive idiotopes.

IX. Antiidiotypic Therapy in Human Tumors

A. B Cell Tumors

Human B cell malignant tumors result from the proliferation of single clones of cells that express surface markers characteristic of normal B-lymphocytes. In particular, the surface immunoglobulin expressed by these cells is monoclonal, i.e., restricted to a single light chain type and to a particular variable region unique to each case. The unique immunoglobulin variable region (idiotype) of each lymphoma clone may be considered a tumor-specif-

ic marker, distinguishing tumor cells from normal cells in the patient. Because of this and because of the malignant course of such tumors it is not surprising that the first attempts at therapeutic intervention with anti-idiotypic antibody have taken place in the context of treating patients with B cell tumors. In 1980 Hamblin *et al.* have described a patient with chronic lymphocytic leukemia who was treated with polyclonal sheep antiidiotype globulin. In that case, treatment produced a transient fall in circulating leukemia cells but was associated with considerable toxicity, including fever, rigors, and respiratory distress. In 1982 Miller *et al.* treated a patient suffering from B cell lymphoma with a mouse monoclonal antiidiotype antibody which reacted solely with the patient's B cells. The patient was repeatedly plasmapheresed in an attempt to reduce the level of circulating idiotype. Large doses of antiidiotype (150 mg) were given intravenously. Anti-Id was demonstrated bound to the surface of malignant cells obtained from lymph nodes. Administration of the antiidiotype was followed by remission which persisted well beyond the period of passive therapy and was characterized by lack of acute or chronic toxicity. The mechanisms of the action of anti-Id antibody in inducing remission of the B cell tumor was not clearly established. Possible mechanisms of direct antibody-mediating killing *in vivo* include complement-dependent lysis, antibody-dependent cell-mediated cytotoxicity, and phagocytosis of antibody-coated cells by the reticuloendothelial system. Of these possibilities a role for hemolytic complement is unlikely because of the difficulty in demonstrating lytic effects of mouse antibodies on human cells *in vitro* using human complement and because serum hemolytic complement in patients undergoing antibody therapy is not decreased. Indirect mechanisms could be postulated to explain the therapeutic effect of the antiidiotype antibody. It is possible that the antiidiotype antibody triggers an active antitumor response which could outlast the direct effects of passively administered antibody. This may explain why a remission can occur 3–4 months after administration of anti-Id and why such remissions persist.

Following the initial report of the success of monoclonal anti-Id in inducing complete remission of a B cell follicular lymphoma, further attempts at passive therapy with anti-Id have been less successful. The lack of therapeutic response of B cell neoplasms may be a consequence of several obstacles associated with antiidiotype therapy. These include, for example, blockade by secreted idiotype in the circulation, modulation of antibody from the cell surface, failure to elicit effective cytotoxicity despite antibody binding, or the occurrence of multiple clones in a single patient. In addition a neoplasm can escape antiidiotype detection though the evolution of a subclone, which, although genetically derived from the parent neoplasm and similar in all respects to it, synthesizes an altered idiotype. This change renders the immunoglobulin invisible to antiidiotype antibody, thereby preventing de-

tection and potential eradication by passive immunotherapy. This phenomenon has been recently well documented in one patient by Raffeld *et al.* (1985) and in two additional patients by Meeker *et al.* (1985). The spontaneous change of idiotype became evident during a regrowth of the lymphoma shortly after an abrupt tumor regression. In this regard, it is intriguing to speculate that a change of idiotype in a normal B cell clone permitted in the first place the neoplasm to elude a previously effective, idiotype-specific host immune response.

Loss of reactivity with monoclonal antiidiotype antibody during therapy with that antibody was not due to antigenic modulation, because although reactivity with antiidiotype antibody disappeared from a population of tumor cells, the cell surface immunoglobulin remained intense and uniform for all cells within the tumor (Fig. 11). Furthermore, modulation would have been reversible when cells were cultured *in vitro* in the absence of the appropriate antibody which was not the case. Furthermore in one patient additional

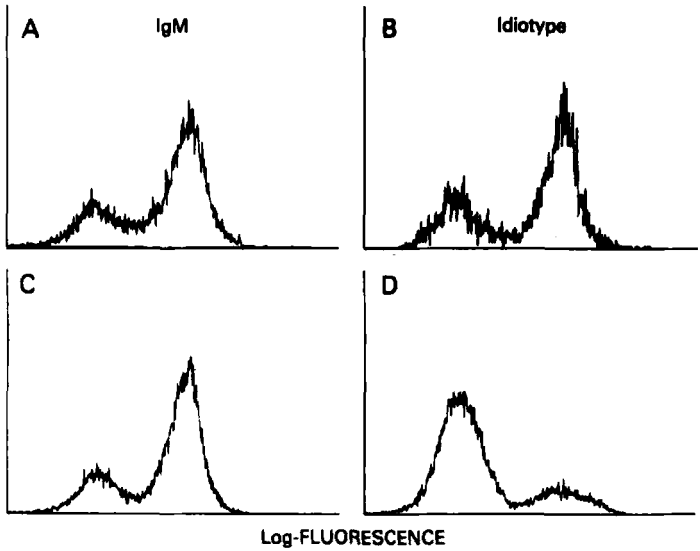

FIG. 11. *Cells from two successive biopsy specimens, obtained immediately before spontaneous tumor regression (A,B) and at relapse 4 months later (C,D), were assayed by indirect immunofluorescence with either monoclonal antihuman IgM (μ chain) (A,C) or antiidiotype (B,D). Approximately equal percentages of cells from the earlier biopsy specimen stained with both anti-IgM (A) (77%) and antiidiotype (B) (74%). The subsequent biopsy specimen contained a monoclonal population composed of 72% IgM-positive cells (C) and 73% κ-positive and less than 1% λ-positive cells (not shown). However, only 22% of the cells from this latter biopsy specimen were stained by antiidiotype (D). Although the amount (fluorescence intensity) of surface IgM per cell was uniform and remained unchanged, the majority of lymphoma cells from the biopsy specimen taken during relapse no longer expressed the original idiotype. (From N. Engl. J. Med.* **312**, 1655, 1985.)

biopsies 3 months after antibody therapy had been stopped still revealed a population of tumor cells that was negative for the idiotype. Therefore, antigenic modulation was extremely unlikely.

Patients with B cell lymphoma may have a tumor cell population that fails to react with an antiidiotype antibody because of the coexistence within the tumor of a totally separate clone of neoplastic B cells (Sklar *et al.*, 1984). In contrast to such biclonal lymphomas, the tumors in the patients studied were found to have the same pattern of immunoglobulin gene rearrangements in all samples studied. This finding strongly suggests that all cell populations studied were part of a single monoclonal lymphoma in each patient.

The loss of idiotype in the patients studied must have resulted from a structural alteration in the surface immunoglobulin molecules. This would include a change in glycosylation and/or a change in the amino acid sequence. Two-dimensional electrophoresis, however, showed no differences among immunoglobulin molecules in specimens obtained at the relevant points in time making it very unlikely that a change in glycosylation has occurred. Furthermore if amino acid changes had occurred in the protein sequence, they did not alter the net charge of heavy or light chain.

A subtle change in the variable-region amino acid sequence probably accounted for the loss of reactivity with the antiidiotype antibody. The DNA encoding the variable region of the immunoglobulin molecule is known to contain sites of frequent mutation. In fact, mutations in this region represent one mechanism for the generation of antibody diversity in normal B cell clones. Studies of myeloma or hybridoma cells that have lost reactivity with antiidiotypic antibodies have demonstrated the replacement of whole segments of the variable-region DNA sequence with those of neighboring variable regions, as well as more subtle events such as point mutations (Baltimore, 1981; Dildrop *et al.*, 1982).

Idiotype mutants thus present obvious difficulties for immunotherapy directed at the idiotype. The magnitude of this problem is not yet clear. Idiotope-negative populations of cells have emerged in patients over a period of only 1 to 2 months during therapy with antibody and in the context of an initial tumor regression. The rapid appearance of sizable idiotope-negative populations of tumor cells after antiidiotype therapy raises an important question concerning the proportion of cells with idiotypic variation that exists in two patients before therapy. The mutation rate in the whole immunoglobulin variable region of a normal B cell clone responding to antigen *in vivo* has been estimated to be as high as 10^{-3} per base pair per cell division (McKean *et al.*, 1984). Similarly, myeloma cell populations are known to accumulate mutations in their immunoglobulin variable regions. In the absence of an antigenic selective force, the prevalence of myeloma cells ex-

pressing variants for any particular idiotypic determinant *in vitro* has ranged from 10^{-8} to 10^{-2} in different cell lines (Bruggemann *et al.*, 1982). The frequency of idiotypic variants in the pretreatment of cell populations of patients is not known. Fluorescence analysis showed complete reactivity with antiidiotype antibody in the pretreatment samples. However, this analysis could have missed up to 1% of idiotope-negative cells. The rapidity of their emergence suggests that the prevalence of idiotype variants was closer to 10^{-2} than to 10^{-8}.

The factors that may increase the prevalence of idiotype variants are not known. Potentially mutagenic chemotherapy could have contributed to a high frequency of variable-region mutations. In some patients selective forces such as endogenous antiidiotypic immune responses or undiscovered antigens with which tumor cells interact may favor the growth of tumor cells that show mutational differences. In the future it would seem appropriate to employ several different antiidiotypic antibodies, each directed at a different idiotope. This approach should reduce the probability of tumor escape due to idiotype variation.

B. T Cell Leukemias

Monoclonal antiidiotypic antibodies to T cell leukemia are of special interest in the treatment of these malignancies. These antibodies react with molecules which are heterodimers and which are present on the immunizing cell and not on normal T cells or other leukemic T cells. One of these anti T cell anti-Id antibodies (Su) has been studied in special detail (Kunkel, 1984). It reacted with a molecule of approximately 80,000 Da which on reduction gave two bands of approximately 43,000 and 38,000 Da. A second monoclonal antibody to the above Su leukemic cells is of special interest because in addition to having specificity for the immunizing cell it showed reactivity with a small population of normal T cells, approximately 2% in all normal individuals. Removal of the surface antigen with either antibody removes reactivity with the other. The antigen recognized by both antibodies is modulated by T3 or Leu-4 T cell antibodies. Thus, it appears that the two antibodies reacted with the same molecules but at different sites. It seems likely that one antibody sees a private idiotype and the second probably was a cross-reactive idiotype. None of the two antibodies reacted with B cells or immunoglobulins. Private idiotype monoclonal antibodies of this type may in the future prove useful in the therapy of these T cell leukemias.

C. B Cell Tumors, Anti-Id, and Acquired C1 Esterase Inhibitor Deficiency

A potential complication of B cell tumor therapy with antiidiotypic antibody could be the development of the syndrome of acquired C1 esterase

TABLE IV
REACTIVITY OF SERUM IgG WITH AUTOLOGOUS B CELLS FROM A PATIENT WITH ACQUIRED C1 ESTERASE DEFICIENCY AND CHRONIC LYMPHOCYTIC LEUKEMIA AND FROM A NORMAL SUBJECT

Source of B cells	Trypsin treatment	Surface isotype: Positive cells (%) with Anti-μ	Surface isotype: Positive cells (%) with Anti-γ	Antiidiotype reactivity: Positive cells (%) with Patient IgG: Total	Patient IgG: Δ	Normal IgG: Total	Normal IgG: Δ
Patient	−	73	16	53	37	18	2
	+	25	12	70	58	14	2
Normal subject	−	70	7	12	5	7	0

inhibitor deficiency and angioneurotic edema. This syndrome has been described in adult patients suffering from a variety of B cell malignancies (Caldwell *et al.*, 1972; Gelfand *et al.*, 1969). It is characterized by decreased levels of C1 esterase inhibitor C1, C2, and C4 but with normal levels of C3. Antiidiotypic antibodies directed against circulating monoclonal immunoglobulins present on the surface of the B cell tumors (Table IV) and in the serum (Fig. 12) have been demonstrated in this syndrome (Geha *et al.*, 1985). The interaction of anti-Id with the idiotype leads to deposition of C1q on the surface of the B cells (Fig. 13).

C1 inhibitor binds covalently to C1r and C1s and dissociates them from C1q. This disassembly of macromolecular C1 results in the formation of C1r–C1s–C1–inhibitor complexes, which have been detected in the serum of some patients, including patients with acquired C1-inhibitor deficiency. The rate of C1-inhibitor consumption in these patients is almost twice the rate in normal controls of in patients with hereditary angioedema.

The activation of C4 and C2 in patients with acquired angioedema and C1-inhibitor deficiency does not result in the formation of an effective classical pathway C3 convertase (C4b2a), and consequently C3 and all the later-acting components are present in normal amounts in the serum of these patients. This aberrant fixation of complement by the idiotype–antiidiotype complex may be due to one or more of several factors. It is apparent that in some cases the ratio of idiotype to antiidiotype is very high and the reaction is well into the zone of antigen excess. Under such circumstances it might be expected from studies with IgG oligomers that the kinetics of C1 fixation would be relatively slow (Tschopp, 1982; Fust *et al.*, 1978). Since the rate of C1–C1–inhibitor interaction is very rapid C1 inhibitor may be preferentially

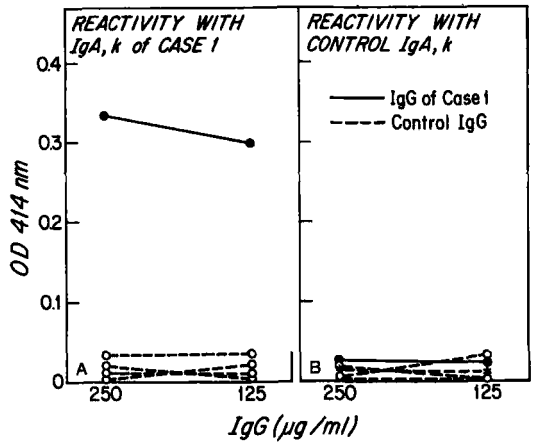

FIG. 12. Reactivity of serum IgG from patient 1 and from four control patients, who had IgA multiple myeloma and normal levels of C1 inhibitor, with IgA M components isolated from patient 1 (A) and from one of the control patients (B). The asterisks at the bottom of B identify the control patient whose IgA was used. Results similar to those shown in B were obtained when IgA M components from the other three control patients were studied. (From *N. Engl. J. Med.* **312**, 536, 1985.)

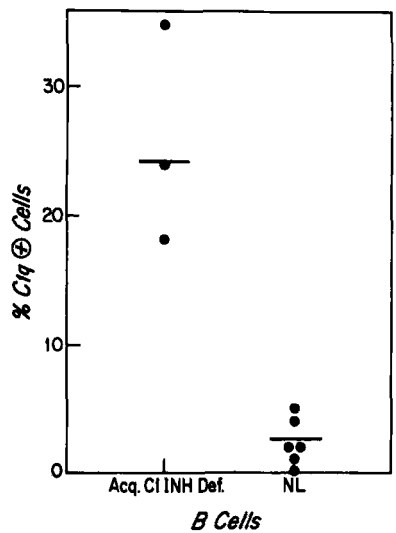

FIG. 13. Percentage of circulating E-rosette-negative (B) cells bearing C1q, as determined by indirect immunofluorescence with mouse monoclonal antibody to C1q and goat anti-mouse IgG-FITC (fluorescein isothiocyanate). Background staining of B cells with mouse ascites and goat anti-mouse IgG-FITC was always less than 5% and was subtracted from the experimental value. (From *N. Engl. J. Med.* **312**, 539, 1985.)

consumed (Ziccardi, 1982). However, since these patients have very low levels of C4, such slow C1 activation does not appear to be a sufficient explanation for the findings observed. It is possible that the stereochemistry of Fab–Fab interactions, as in an idiotype–antiidiotype complex, could impede the formation of C4b2a complexes or render them exquisitely susceptible to attack by the control proteins, C4b-binding protein, and C3b/C4b inactivator (factor I), so that rapid decay of the classical pathway C3 convertase C4b2a is induced. It is also possible that regulatory elements integral to the B cell membrane, where idiotype–antiidiotype interactions take place, impede the formation of C3 convertase (Iida and Nuzzensweig, 1983). The outcome of these events is the failure of C3 activation.

D. Anti-Id Therapy in Solid Tumors

The emergence of a tumor cell often reflects the failure of the immune system to eliminate tumor antigen bearing cells. Induction of anti-Id by mouse monoclonal antibody to tumor antigens may give rise to anti-Id antibodies which bear the internal image of antigen. Such antibodies may elicit an immune response by the host which results in the destruction of tumor antigen-bearing cells. When an antitumor effect occurs a long time after the administration of mAb (Miller *et al.*, 1982), it may be that an immune response of the host to his tumor was induced by mAb treatment.

Koprowski *et al.* (1984) have studied the effect of administration of mouse mAb to colorectal carcinoma antigen (mAb171-A) to patients with gastrointestinal tumors. They found that tumors from several patients regressed after a single administration of mAb171-A. Although mAb may directly destroy tumor cells by activation of complement or by interaction with macrophages or killer cells, it is likely that other mechanisms may have been operative because of the need for very large amounts of mAb to interact with all antigenic sites of target cells of a solid tumor mass and because of the long induction period to measure the antitumor effect. In fact, the decrease in size and eventual disappearance of lung metastases, which could be measured by radiography, occurred over 3–4 months in one patient. Furthermore binding of mAb to metastatic tumor biopsy samples was detected only up to 1 week after injection of mAb.

Evidence for an alternative mechanism involving a network of interaction antiidiotypic T and B cells directed against the mAb was the presence in the patients' serum immunoglobulin fraction of anti-Id antibodies which directly bound ^{125}I-labeled mAb17-1A. This binding was hapten inhibitable, i.e., it was inhibited by a 3 *M* KCl cell extract of a human colon carcinoma. This hapten inhibition of the binding reaction suggests the presence of an "internal image" of the tumor epitope on the anti-Id molecules. All human anti-Ids studied showed cross-reactivity. Thus it seems likely that the inoculation of

mAb 17-1A in other subjects could induce production of cross-reacting anti-Ids and possibly identical internal images of the cancer antigen.

The presence of an internal image of an antigen on a human immunoglobulin molecule, in contrast to the presence of the antigen on a tumor cell, may change the conditions under which the immune system reacts to the tumor antigen. Although it is still difficult to correlate the presence of anti-Id with the outcome of the immunotherapy, three out of five patients who produced anti-Id have had tumor regression resulting in no detectable disease. The most direct evidence for a beneficial effect of anti-Id would be the administration of anti-Id, produced in a patient, to a nonresponsive cancer patient and the triggering of an antitumor response, which could be measured *in vivo* and *in vitro*.

X. Conclusion

There is little doubt that idiotypic interactions play a role in the regulation of the human immune response in health and disease. It is also evident that idiotypic interactions can be manipulated to the advantage of the host in the treatment of human disease. Treatment of human B cell tumors with anti-Id antibodies is already a reality. Vaccination with anti-Id and prevention of allograft rejection are therapeutic strategies that are successful in experimental animals but remain untried in man.

Several problems must be considered in the manipulation of idiotypic interactions for the treatment of human disease.

1. In the case of antitumor therapy for B cell/T cell tumors, several anti-Id monoclonal antibodies directed to different idiotopes should be used to minimize tumor cell "escape" by mutations. This would involve a substantial effort since anti-Id must be tailored to each patient.

2. Repeated treatments with heterologous anti-Id may give rise to anti-isotype antibodies which can lead to rapid elimination of the antiidiotype. The use of antibodies made by human B–B hybridomas will alleviate this problem.

3. In the case of allograft rejection and autoimmune disease administration of anti-Id antibodies relies on the presence of dominant cross-reactive idiotypes (CRI). However the suppression of major CRI may be accompanied by an increase in the level of non-CRI. In the antibody response to TT, booster immunization results in a rise in total antibody titer to TT in the face of a decrease in the level of idiotypes present at or shortly after immunization due to modulation by auto-anti-Id. The compensatory rise in the levels of nonsuppressed idiotypes may diminish the efficacy of treatment.

4. The suppression of undesirable antibodies, e.g., antiself by anti-Id will

result in the suppression of antibodies of other specificities which share the idiotype being suppressed. The resultant holes in the repertoire may be significant enough to decrease the defense of the host against foreign organisms.

5. Complications due to the presence of idiotype anti-Id complexes *in vivo* are potentially many and could be serious (e.g., immune complex disease, acquired C1 esterase inhibitor deficiency) and must be monitored carefully.

6. The safety of the reagents (anti-Id or other mAb) to be used must be established by screening for viruses, oncogens, and other agents.

Despite these reservations the use of anti-Id antibodies in the treatment of human disease is an exciting and challenging field. While now restricted to malignant conditions it is likely that their next use will be as vaccines against diseases such as schistosomiasis and trypanosamiasis. In addition to their mortality these two diseases have high morbidity and a profound detrimental effect on the economy of underdeveloped countries. This greatly contributes to a poor standard of living and to death from other diseases including malnutrition. Vaccination with anti-Id antiidiotypic antibodies bearing the internal image of antigen would easily outweigh the risks involved. Finally the use of anti-Id in the therapy of human diseases should incite an expansion of our knowledge of the role played by idiotypic interactions in human health and disease. The next few years promise to be exciting for workers in this field.

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ADVANCES IN IMMUNOLOGY, VOL. 39

Neuroimmunology

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I. Introduction

It has been apparent for many decades that bidirectional communication between the immune and nervous systems provides unique opportunities for coordinate mobilization of the specialized capacities of each system to sense and respond to environmental and autologous challenges (Fig. 1) (Ader, 1981; Spector, 1983; Guillemin *et al.*, 1985; Goetzl, 1985). Despite the many exciting possibilities for greater understanding of neuroendocrine and immune interactions in host defense and disease, only recently has this complex field of research yielded to applications of modern molecular and cellular biology. Although the goals of studies of neuroimmunology are to elucidate the bases of integrated physiological events, most of the tangible accomplishments have been limited to analyses of the neuroanatomy of organs of immunity, the antigens, receptors, and synthetic pathways shared by the two systems, the interdependence of their development, and functional interactions (Figs. 2 and 3). The effects of behavior as a set of variables that conditions neuroimmunological communication have just begun to be explored meaningfully.

Electrolytic lesions of the hypothalamus, hippocampus, and some adjacent structures of rodents result in alterations in the number and functions of natural killer (NK) cells and T-lymphocytes in the spleen, thymus, and blood, in a manner determined by cerebral asymmetry and age (Jankovic and Isakovic, 1973; Stein *et al.*, 1976; Brooks *et al.*, 1982; Renoux *et al.*, 1983; Cross *et al.*, 1984a,b). The neuroanatomical bases for some of the observed immunological effects of central nervous system (CNS) lesions appear to be alterations in specific noradrenergic and peptidergic fibers that end in zones of lymphocytes in thymus, spleen, Peyer's patches, and bone marrow (Bulloch and Pomerantz, 1984; Felten *et al.*, 1985). The development of the thymus in some species also is dependent on the integrity of distinct sections of neural crest (Bockman, 1984) and neuroendocrine functions (Pierpaoli *et al.*, 1976).

Protein antigens shared by lymphocytes and neural cells include the Leu-7 glycoprotein antigen, that is found broadly in natural killer (NK) cells,

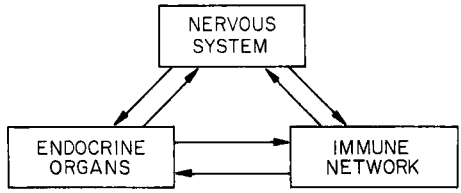

FIG. 1. Communication in the neuroendocrine–immune networks.

some other cytotoxic lymphocytes, neuroectodermal cells in the CNS and peripheral nervous system (PNS) (Lipinski *et al.*, 1983; Schuller-Petrovic *et al.*, 1983), glycoproteins associated with myelin (Inuzuka *et al.*, 1984), and some fetal and neoplastic neuroendocrine cells (Bunn *et al.*, 1985); the Thy-1 surface glycoprotein of rodent thymocytes and neurons (Tse *et al.*, 1985); and the mouse H-2 and Ia antigens and human HLA and β_2-microglobulin on macrophages, lymphocytes, and neural cells of the respective species (Lampson and Fisher, 1984; Wong *et al.*, 1984). The functional relevance of the presence of shared antigens is suggested by the capacity of anti-Thy-1 to stimulate both proliferation of mouse T-lymphocytes (Maino *et al.*, 1981; Gunter *et al.*, 1984) and neuronal outgrowth (Leifer *et al.*, 1984), and of γ-interferon to increase the expression of class I and II histocompatibility antigens on mouse and human neurons, as well as immune cells (Lampson and Fisher, 1984; Wong *et al.*, 1984). Chromogranin, a secretory protein marker of neuroendocrine cells, has been demonstrated immunochemically in rodent spleen, lymph nodes, and thymus (Angeletti and Hickey, 1985).

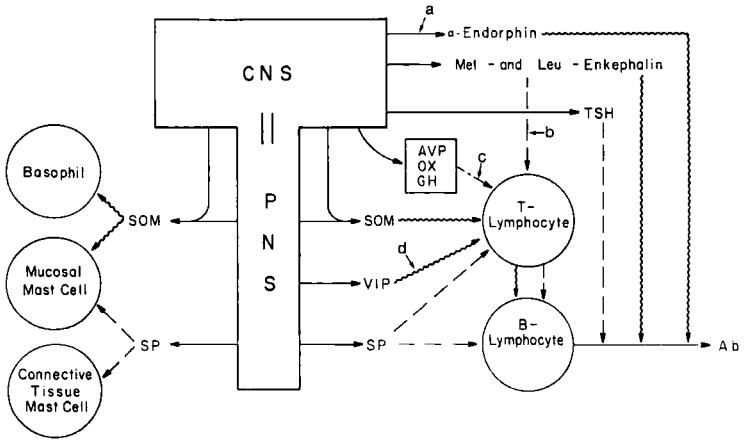

FIG. 2. Immunological effects of neuroendocrine mediators. CNS, Central nervous system; PNS, peripheral nervous system; Ab, antibody; GH, growth hormone; AVP, arginine vasopressin; OX, oxytocin. (a) Generation or release, (b) stimulation, (c) enhancement, (d) inhibition.

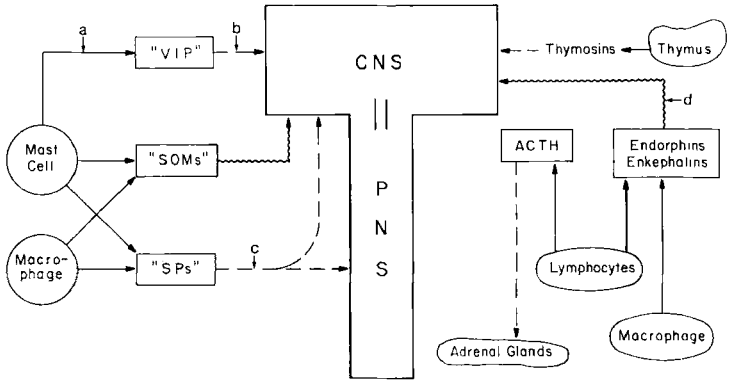

FIG. 3. Neuroendocrine effects of immunological mediators. CNS, Central nervous system; PNS, peripheral nervous system; Ab, antibody; GH, growth hormone; AVP, arginine vasopressin; OX, oxytocin. (a) Generation or release, (b) stimulation, (c) enhancement, (d) inhibition.

Although initially considered to be mediators exclusive to immunity, interleukins have been shown recently to be produced and recognized by cells of the nervous system. Glial cells secrete interleukin 1 (Fontana *et al.*, 1982, 1984) and astrocytes generate interleukin 1 (Fontana *et al.*, 1982, 1983) and interleukin 3 (Frei *et al.*, 1985). Interleukin 1 and related factors stimulate the proliferation of both astroglia and oligodendroglia (Merrill *et al.*, 1984; Giulian and Lachman, 1985). A fascinating recent development is the finding that interleukin 1 may mimic the effects of corticotropin-releasing factor (CRF) by evoking the release of ACTH from cultured mouse pituitary cells (Woloski *et al.*, 1985).

The principal focus of this article will be the peptide mediators generated and recognized by distinct elements of the neuroendocrine-immune network, that govern many critical developmental and functional interactions (Figs. 2 and 3). The results of numerous studies suggest that behavioral conditioning, stress, exercise, and sexual activity can have major effects on immunity (Ader, 1981; MacLean and Reichlin, 1981; Spector, 1983; Shavit *et al.*, 1984; Russell *et al.*, 1984; Goetzl, 1985; Ahmed *et al.*, 1985), but the methods and interpretations are sufficiently different from other approaches in neuroimmunology as to preclude consideration in the present discussion.

II. Physiological and Pathophysiological Functions of Neuropeptides in Nonneural Tissues

The potent effects of neuropeptides observed in many organ systems suggest the possibility of critical contributions to the physiology of homeostasis and host defense, as well as to the altered tissue reactions characteristic of

diverse diseases. The specific focus of this section will be the direct actions of neuropeptides on target tissues, that are independent of the participation of mast cells, macrophages, lymphocytes, and other cells committed to the immune system. For the present emphasis on interactions between the nervous and immune systems, considerations of the activities of neuropeptides on nonneural tissues will be restricted to those that either resemble actions of immunologically derived mediators or modify the net consequences of immune responses. For example, the smooth muscle, epithelial cell, and vascular activities of substance P (SP), somatostatin (SOM), and vasoactive intestinal peptide (VIP), that resemble or antagonize those of mediators generated by mast cells, will be reviewed to the exclusion of the solely neuroendocrine effects of the peptides.

Peptides released from peripheral nerve endings in mammals, including SP, other tachykinins, and VIP, are among the most potent mediators of smooth muscle and vascular functions (Table I). SP contracts strips of guinea

TABLE I
DIRECT EFFECTS OF NEUROPEPTIDES ON NONNEURAL TISSUES

Action	Target tissues	Mediators
Smooth muscle contraction	Intestines	SP[a], OP[b]
	Esophagus	VIP
	Pulmonary airways	SP, BO, GRP, CGRP, EH
Smooth muscle relaxation	Intestines	VIP
	Lower esophageal sphincter	VIP
	Pulmonary airways	VIP
Vasodilatation	Cutaneous microvessels	VIP
	Systemic arterioles	SP
	Cerebral arteries	VIP
Vasoconstriction	Cerebral arteries	SP
Increased microvascular permeability	Skin	SP
Increased secretion	Salivary glands	VIP
	Tracheal epithelium	SP, VIP
	Nasal epithelium	SP
Decreased secretion	Pancreatic islets	SOM
	Gastric parietal cells	SOM

[a] SP, Substance P; OP, endogenous opioid peptides; VIP, vasoactive intestinal peptide; BO, bombesin; GRP, gastrin-releasing peptide; CGRP, calcitonin gene-related peptide; EH, enkephalin heptapeptide; SOM, somatostatin.

[b] Although β-endorphin and the enkephalins suppress electrically induced contractions of smooth muscle from the gastrointestinal tract and other sources, the primary effects of endogenous opioids and synthetic analogs are contraction of isolated strips of intestine *in vitro* and increases in tone, without directed motility, of intact segments of bowel *in vivo*.

pig ileum, with a 50% effective concentration (EC_{50}) of $1–2 \times 10^{-9}$ *M*, as well as smooth muscle from other sources (Pernow, 1983). In contrast, VIP relaxes intestinal smooth muscle with an EC_{50} of 2.5×10^{-8} *M* for guinea pig ileal tissue (Said, 1982a). The effect of 10–100 n*M* VIP may exhibit anatomical specificity, as the body of the esophagus responds with contraction and the lower esophageal sphincter with relaxation (Biancani *et al.*, 1984). Numerous neuropeptides, including SP, bombesin, gastrin-releasing peptide (GRP), that is the mammalian equivalent of bombesin, calcitonin gene-related peptide (CGRP), and a naturally occurring heptapeptide analog of met-enkephalin constrict pulmonary airways *in vivo* and *in vitro* (Impicciatore and Bertaccini, 1973; Lundberg *et al.*, 1983, 1985; Chipkin and Chapman, 1984; Karlsson *et al.*, 1984). As in many of the other systems, VIP has actions opposite to those of SP and dilates pulmonary airways, as well as reversing the constriction evoked by histamine (Said, 1982b). The principal vascular activity of the neuropeptides is dilatation, that is evident in systemic arteries and arterioles for SP (Pernow, 1983), and the cerebral arteries and cutaneous microvasculature for VIP (Lee *et al.*, 1984). SP has been observed to constrict the cerebral arteries of some species of mammals, although it is a less reproducible effect than the dilatation elicited by VIP (Lee *et al.*, 1984). The striking vasodilatory activity of SP leads to profound hypotension after administration *in vivo* (Pernow, 1983).

The capacity of epithelial cells and glands to secrete a wide range of polypeptide hormones, glycoproteins, and electrolytes is subject to regulation by neuropeptides (Table I). The flow of electrolytes and fluid from salivary glands is augmented by VIP (Lundberg *et al.*, 1982). The local application of SP enhances nasal secretions in humans and rats (Bernstein and Hamill, 1981; Lundblad *et al.*, 1983). Human, dog, and ferret tracheal epithelial and glandular secretion of glycoprotein-rich fluid is enhanced by SP, related tachykinins, and VIP (Borson *et al.*, 1986). SP and related tachykinins, but not VIP, are more effectively potent as respiratory secretagogues in the presence of inhibitors of enkephalinases. Although initially characterized in relation to its ability to inhibit the secretion of growth hormone by the pituitary, SOM is distributed widely in neuroendocrine and gastrointestinal tissues and suppresses the secretion of insulin and glucagon by the pancreas (Brown *et al.*, 1976), and of hydrochloric acid and pepsin by the stomach (Gomez-Pan *et al.*, 1975). The depletion or inactivation of local stores of SOM augments secretion by the respective glands and cells (Patel *et al.*, 1985). In addition, SOM stimulates the absorption of chloride by small and large intestines and thus regulates the elimination of ions by the gut (Rosenthal *et al.*, 1983).

The attribution of physiological or pathophysiological significance to the experimentally observed effects of neuropeptides in any system at least

requires evidence of local availability at functionally relevant concentrations and of stereospecific receptors in the target tissues of the same species. Much of the work with neuropeptides of the afferent and central nervous system has been carried out with rats, guinea pigs, and rabbits, and only to a much lesser extent with humans. Physiological systems for producing and delivering some of the neuropeptides to sites of purported actions have only been elucidated partially. For example, the finding of a substantial quantity of enkephalin heptapeptide (EH), but not met-enkephalin, in pulmonary airway tissues (Tang *et al.*, 1982) supports the possibility of an important role for EH in bronchospasm. Similarly, a contribution of VIP to cerebral vasodilatation is more likely in view of the presence of VIP in specific nerve endings of the walls of cerebral arteries (Edvinsson *et al.*, 1981). The recent demonstration of SP in nasal secretions after local antigen challenge also lends credence to a role for SP in allergic reactions. Further qualifications of the acceptance of apparently direct actions of some neuropeptides have come from the discovery of contributions of second mediators. The dilatation of human cutaneous microvasculature by SP, neurotensin (NT), dynorphin, and β-endorphin is largely a function of histamine release from mast cells activated by the neuropeptides (Goetzl *et al.*, 1985a). Similar effects of SOM, and to some extent VIP, on cutaneous microvessels results from stimulation of the release of SP from local primary afferent nerve endings (Anand *et al.*, 1983). In other systems, SOM inhibits the release of SP from nerve endings (Gazelius *et al.*, 1981). The use of inhibitors of generation and antagonists of action of second mediators has corroborated their roles in some cases. Examples include suppression of the bronchospastic action of EH by inhibitors of thromboxane synthetase (Chipkin and Chapman, 1984) and elimination of the cutaneous vasodilatory effect of SP by H1-type antihistamines (Foreman and Jordan, 1983). The complex methods required to provide evidence of local secretion and direct action of neuropeptides, and the lack of availability of specific receptor antagonists for many neuropeptides have prevented definition of their roles in some physiologic processes and pathophysiologic states.

III. Mediation and Modulation of Immediate Hypersensitivity

The local tissue concentrations of some neuropeptides are elevated in relation to immediate hypersensitivity reactions, and achieve levels that are sufficient both to exert direct effects on tissues and to influence mast cells and basophils. Subsequent experiments have been designed to elucidate the mechanisms and significance of neuropeptides in immediate hypersensitivity. Diverse peptides of both the CNS and PNS stimulate mast cells to release histamine and leukotrienes *in vitro* (Fig. 4). That micromolar or

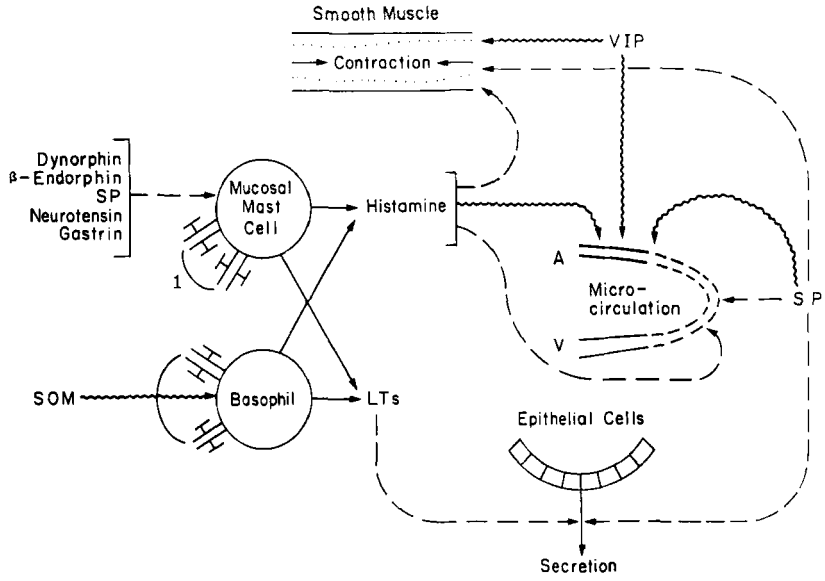

FIG. 4. Regulation of immediate hypersensitivity by neuropeptides. A, arteriole; V, venule; 1, IgE; LTs, leukotrienes.

greater concentrations of SP, SOM, NT, and many other neuropeptides were required to evoke substantial release of histamine by rat serosal or connective tissue-type mast cells (Theoharides and Douglas, 1981; Foreman *et al.*, 1982; Foreman and Jordan, 1983) suggested that the effect might be simply analogous to that of other chemically basic factors (Baxter and Adamik, 1978; Brindley *et al.*, 1983). However, unlike other basic peptides, the activity of SP exhibited cell specificity and included stimulation of the generation of unstored mediators, such as leukotrienes. Mouse mast cells derived from bone marrow precursors under the influence of interleukin 3, that serve as a model for mucosal-type mast cells, are activated to release histamine, LTC_4, and LTB_4 by 1/1000–1/100 the concentration of SP required to release histamine from connective tissue-type mast cells (Goetzl *et al.*, 1986). In contrast, basophils fail to respond *in vitro* to SP or other neuropeptides at concentrations as high as 10^{-5} *M* (Goetzl and Payan, 1984). The mast cell responses elicited by SP were distinguished clearly from those dependent on IgE by the apparent lack of a requirement for extracellular calcium and of any effect of prior desensitization that prevents activation by IgE-related mechanisms (Foreman and Jordan, 1983).

SOM expresses only minimal mast cell-activating and no significant basophil-activating activity, but selectively inhibits the IgE-dependent stim-

ulation of basophils to release histamine and other mediators (Fig. 4) (Goetzl and Payan, 1984). The release of histamine and LTD_4 from human basophils and rat leukemic basophils, challenged respectively with anti-human IgE and anti-rat $F(ab')_2$, was inhibited by picomolar to nanomolar concentrations of SOM. In contrast, SOM failed to inhibit a similar level of release of mediators from basophils stimulated by ionophore A23187, suggesting a specificity of SOM for immunological mechanisms of activation. Further evidence for the specificity of the SOM effect on basophils was derived from the dependence on peptide conformation, that was suggested by the lower potency of reduced and alkylated SOM and the absence of activity of [D-Trp^8] SOM (Goetzl and Payan, 1984). Finally, the lack of inhibitory effect of SOM on mediator release from rat serosal mast cells and the requirement for 1,000- to 10,000-fold higher concentrations of SOM for inhibition of the release of mediators by mouse mucosal mast cells than by basophils (Goetzl *et al.*, 1986) attested to the target cellular specificity of this neuroimmunological control.

Thus, although some neuropeptides found principally in the central nervous system have a capacity to degranulate mast cells at high concentrations (Casale *et al.*, 1984), the major direct and indirect neural effects on the expression of immediate hypersensitivity are attributable to peptides released from the peripheral nervous system, including SP, VIP, and SOM (Figs. 2 and 4). SP acts directly on target tissues, to contract smooth muscles, constrict pulmonary airways, increase the permeability of microvasculature, and stimulate epithelial cell secretion in the gut and lungs. SP also acts indirectly by stimulating the release of mediators from mast cells, and thereby recruiting the smooth muscle contractile, vasoactive, and secretion-enhancing activities of histamine, leukotrienes, and other mediators. The application of substituent peptides of SP and antagonists of second mediators has permitted the definition of two determinants in SP. The carboxy-terminal section accounts for the direct actions and the amino-terminal tetrapeptide for the stimulation of mast cells, that consequently releases mediators of the indirect effects of SP (Mazurek *et al.*, 1981; Foreman and Jordan, 1983). That the cutaneous microvascular dilatory effect of SP is mediated indirectly by mast cell-derived histamine, for example, was confirmed by the similar activity of intact SP and the amino-terminal tetrapeptide and by the selective inhibition achieved by pretreatment with H1-type antihistamines (Foreman and Jordan, 1983). VIP contributes directly by relaxing smooth muscles, especially pulmonary airways, and by dilating the microvasculature of lung and other tissues. SOM acts mainly by the indirect mechanisms of inhibiting the release of mediators from immunologically activated basophils and, at higher concentrations, mucosal mast cells, and thus suppresses and limits in time some immediate hypersensitivity reactions. The potential roles for neuropeptides in immediate hypersensitivity therefore encompass direct tissue

effects, recruitment of mast cell contributions, and inhibition of further release of mediators from basophils and mucosal mast cells.

IV. Inflammation and Tissue Repair

Neuropeptides influence the cellular elements of inflammatory responses and tissue repair processes by stimulating mononuclear and PMN leukocyte chemotaxis, regulating the release of inflammatory mediators from macrophages and lymphocytes, and enhancing the proliferation of fibroblasts, smooth muscle cells, and endothelial cells (Fig. 5). The opioid peptides β-endorphin and met-enkephalin stimulate human mononuclear leukocyte chemotaxis *in vitro* with a bimodal dependence on peptide concentration, that is characterized by maximal activities both at 10^{-12} and 10^{-8} *M* (Van Epps and Saland, 1984). The distance that mononuclear leukocytes migrated in response to optimal concentrations of β-endorphin or met-enkephalin was approximately 80% of that traveled in response to 10^{-8} *M N*-formyl-methionyl-leucyl-phenylalanine (fMLP). The chemotactic response to the opioids, but not fMLP, was blocked selectively by preincubation with 10^{-8} *M*

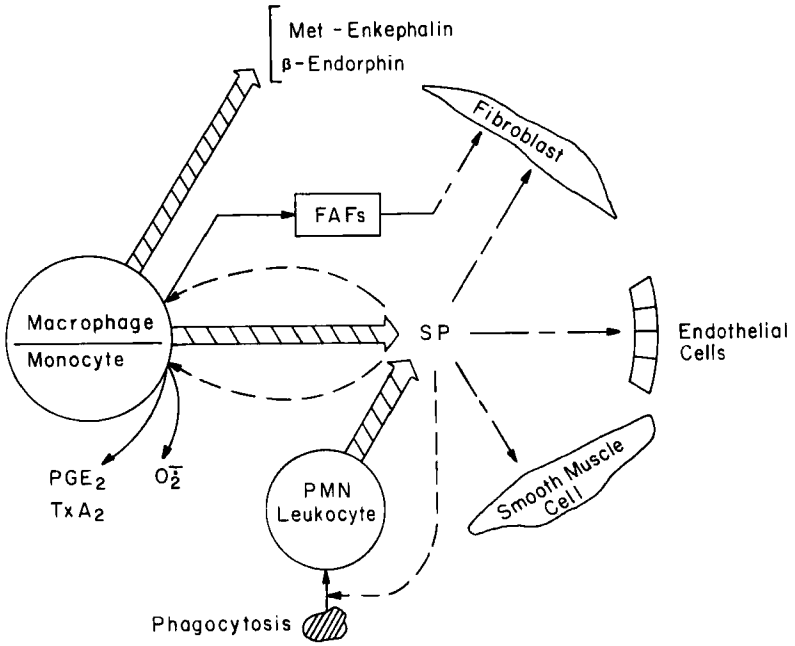

FIG. 5. Effects of neuropeptides on inflammation and tissue repair. PGE_2, Prostaglandin E_2; TxA_2, thromboxane A_2; O_2^-, superoxide anion; FAF, fibroblast-activating factor.

naloxone, suggesting that the opioids are recognized by stereospecific receptors on the leukocytes (Van Epps and Saland, 1984). Human mononuclear leukocyte chemotactic responses also were evoked by neuropeptides of the PNS, such as SP (Ruff *et al.*, 1985). SP stimulates human monocyte chemotaxis *in vitro* with an EC_{50} of approximately 0.1 p*M*. The chemotactic effects of SP were blocked by D-amino analogs of SP, but were not inhibited by fMLP antagonists (Ruff *et al.*, 1985). SP elicits not only mononuclear leukocyte chemotaxis, but also the generation of thromboxane A_2, O_2^-, and H_2O_2 by *C. parvum*-activated macrophages (Hartung and Toyka, 1983). Several other macrophage responses are evoked by SP, including the down-regulation of membrane-associated 5′-nucleotidase and the stimulation of synthesis and release of metabolites of arachidonic acid (Hartung *et al.*, 1986).

The capacity of SP to stimulate chemotactic responses of PMN leukocytes is attributed to binding to the fMLP receptors. However, the EC_{50} of 10 μ*M* SP suggests that this observed effect may not be physiologically significant (Marasco *et al.*, 1981). Other PMN leukocyte functions that are enhanced by nanomolar concentrations of SP include the induction of lysosomal enzyme release (Marasco et al., 1981) and the stimulation of phagocytosis of yeast cells (Bar-Shavit *et al.*, 1980). The leukocyte-directed effects of SP are attributable to distinct molecular domains, as are the effects of SP in immediate hypersensitivity. The chemotactic activity seems to reside in the carboxy-terminal substituent peptide of SP, whereas the phagocytosis-enhancing activity appears to reside in the N-terminal tetrapeptide (Bar-Shavit *et al.*, 1980). The N-terminal tetrapeptide of SP is important for the stability of the native undecapeptide, as it retards the degradation of SP by aminopeptidases (Blumberg and Teichberg, 1979). Recent studies have also demonstrated that β-endorphin and met-enkephalin, but not α-endorphin, inhibit the production by Con A-stimulated peripheral blood mononuclear cells of a specific T-lymphocyte chemotactic factor, that appears not to be related to other leukotactic stimuli (Brown and Van Epps, 1985).

Neuropeptides may regulate the processes of tissue repair by enhancing the proliferation of both smooth muscle cells and fibroblasts (Nilsson *et al.*, 1985; Payan, 1985). The proliferative effect of SP on smooth muscle cells was diminished by preincubation with SP antagonists or simultaneous stimulation with suboptimal concentrations of other growth factors, suggesting the possibility of competition for a common subset of receptors for polypeptide growth factors (Nilsson *et al.*, 1985). That the mitogenic effects of SP on smooth muscle cells are receptor mediated, was demonstrated by saturable specific binding of ^{125}I-labeled SP with a K_D of approximately 0.5 n*M* and a density of 40,000–50,000 receptors/cell. Proliferation-enhancing concentrations of SP increased the concentration of cytosolic Ca^{+2} in fibroblasts,

implying a concurrent activation of the phosphatidylinositol pathway (Payan, 1985). Recent experimental results indicate that fibroblast polypeptide growth factors, capable of expressing potent mitogenic and angiogenic activities, have significant amino acid sequence homologies with neuropeptides of the tachykinin family such as SP, substance K, and neuromedin C (Gimenez-Gallego *et al.*, 1985).

V. Lymphocyte Functions

A wide range of functions of T- and B-lymphocytes and NK cells has been shown to be inhibited or stimulated by neuroendocrine peptides of the CNS, such as β-endorphin, α-endorphin, ACTH, and met- and leu-enkephalins (Johnson *et al.*, 1982; Gilman *et al.*, 1982; McCain *et al.*, 1982; Mathews *et al.*, 1983; Puppo *et al.*, 1985; Heijnen *et al.*, 1986) (Fig. 6). The immunosuppressive properties of α-endorphin were demonstrated initially with murine splenocytes (Johnson *et al.*, 1982). At concentrations as low as 50 n*M*, α-endorphin significantly suppressed the *in vitro* responses of T-lymphocyte-dependent plaque-forming cells (PFC) to sheep red blood cells. Moreover, the inhibitory effects of α-endorphin were blocked by preincubating the splenocytes with either naloxone or β-endorphin, suggesting that the effects of α-endorphin were mediated by an opiate receptor (Johnson *et al.*, 1982). Neither β-endorphin nor γ-endorphin had significant inhibitory effects on

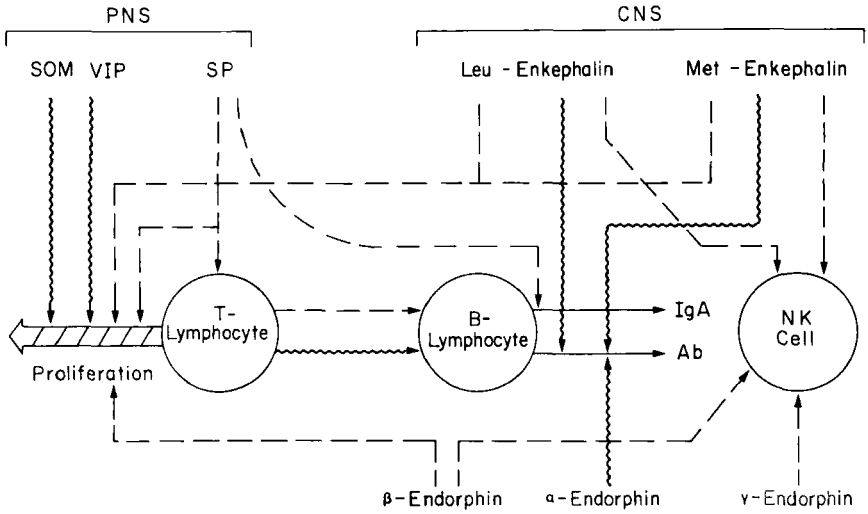

FIG. 6. Modulation of lymphocyte functions by neuropeptides. IgA, Immunoglobulin A; NK, natural killer.

the murine PFC, whereas both leu- and met-enkephalin inhibited the responses at concentrations approximately 10 times greater than that required for α-endorphin (Johnson *et al.*, 1982). The results of such functional studies suggested that recognition of the amino-terminal amino acids and neuropeptide conformation determined the specificity of the receptors for enkephalins and endorphins on murine splenocytes capable of regulating immune responses (Johnson *et al.*, 1982). Recent evidence indicates that α-endorphin also suppresses the primary antibody response of human lymphocytes to ovalbumin (OA) (Heijnen *et al.*, 1986). At concentrations as low as 50 n*M*, α-endorphin blocked the OA-specific IgM-PFC response at both the T- and B-lymphocyte levels. In addition, α-endorphin exhibited the capacity to inhibit the conversion of B-lymphocytes into PFC (Heijnen *et al.*, 1986).

Experiments designed to determine the role of β-endorphin in modulating the contributions of lymphocytes to immunological responses have yielded conflicting results. The proliferative responses of rat splenocytes were stimulated by nanomolar concentrations of β-endorphin (Gilman *et al.*, 1982). The finding that potentiating effects of β-endorphin on T cell responses to concanavalin A (Con A) or phytohemagglutinin (PHA) were not blocked by naloxone implied that β-endorphin was not acting through an opiate-like receptor. In contrast, 10^{-7} *M* β-endorphin inhibited PHA-induced proliferation of human T-lymphocytes by 75%, apparently also by acting through a nonopiate receptor-like mechanism (McCain *et al.*, 1982). Recent results have confirmed the inhibitory effects of β-endorphin on PHA-induced proliferation of human T-lymphocytes, and shown β-endorphin-dependent decreases in the expression of the T_4 and HLA-DR surface antigens on cultured mononuclear leukocytes, as measured with fluorescent monoclonal antibodies directed against these specific membrane antigens (Puppo *et al.*, 1985). β-endorphin and met-enkephalin both enhance human NK cell activity directed against K562 cells. The mechanisms by which β-endorphin increased effective activity of NK cells included a recruitment of effector cells from pre-NK cells, as well as increased recycling of effector cells (Mathews *et al.*, 1983).

Neuropeptides secreted in tissues predominantly by the PNS, such as SP, SOM, and VIP, also modulate both murine and human lymphocyte functions (Payan and Goetzl, 1985) (Fig. 6). Picomolar concentrations of SOM significantly inhibited the proliferation of Molt 4 lymphoblasts and PHA-stimulated human blood T-lymphocytes (Payan *et al.*, 1984a). Other immunomodulatory influences of SOM include the suppression of endotoxin-induced leukocytosis (Wagner *et al.*, 1979) and the inhibition of release of colony-stimulating activity by murine splenic lymphocytes (Hinterberger *et al.*, 1977). Nanomolar concentrations of SOM inhibited the proliferation of murine lymphocytes derived from both spleen and Peyer's patches. In addi-

tion, SOM also inhibited significantly the synthesis of IgA, but not IgM or IgG, by lymphocytes of the spleen and Peyer's patches (Stanisz *et al.*, 1986). The same degree of inhibition of proliferation of murine lymphocytes, as was seen with SOM, also was demonstrated for VIP in lymphocytes from spleen and Peyer's patches (Ottaway and Greenberg, 1984; Stanisz *et al.*, 1986). In contrast to SOM, VIP only influenced immunoglobulin production in murine Peyer's patches where it stimulated the production of IgM and inhibited that of IgA (Stanisz *et al.*, 1986). Biochemical analyses of the interaction of VIP with lymphocytes have shown that VIP activates adenylate cyclase in membrane preparations from Molt 4b lymphoblasts, and thereby increases the concentration of cAMP in the intact cells (Beed *et al.*, 1983). That VIP activates lymphocytes by a cAMP-dependent protein kinase was confirmed by demonstrating the ability of VIP to selectively induce the phosphorylation of a specific 41,000 Da protein (O'Dorisio *et al.*, 1985a). VIP receptors on lymphocytes not only mediate cellular activities, but also influence the distribution of T-lymphocytes in gut-associated lymphoid tissue. Preincubation of rat T-lymphocytes from Peyer's patches with VIP, at concentrations that down-regulate the VIP receptors, eliminates homing and reduces the extent of return of reinjected T-lymphocytes to the Peyer's patches (Ottaway, 1984).

The immunologic properties of SP in contrast to those of SOM and VIP are largely stimulatory. The proliferation of both human (Payan *et al.*, 1983) and murine (Stanisz *et al.*, 1986) T-lymphocytes is significantly stimulated by nanomolar concentrations of SP. In human T-lymphocytes, SP and SP(4–11) stimulated respective increases of 60 and 70% in [^{3}H]thymidine and [^{3}H]leucine uptake *in vitro* in the presence or absence of mitogens (Payan *et al.*, 1983). Similar stimulatory effects on the uptake of [^{3}H]thymidine were observed when SP was incubated with murine lymphocytes from spleen, mesenteric lymph nodes, and Peyer's patches. In addition, SP significantly increased the *in vitro* production of IgA in lymphocytes from spleen and Peyer's patches, by as much as 300% (Stanisz *et al.*, 1986). Biochemical characterization of the actions of SP at the level of lymphocyte membranes have shown that SP stimulates the incorporation of ^{32}P into membrane phospholipids by activating the phosphatidylinositol pathway (McGillis *et al.*, 1986).

VI. Neuropeptide Receptors on Immune Cells

Functionally relevant receptors for neuroendocrine mediators of the CNS have been demonstrated on the surfaces of different types of lymphocytes. Receptors for leu-enkephalin on Jurkat T-lymphocytes have a K_D of 2.03×10^{-6} *M*, as determined by direct binding assays (Ausiello and Roda, 1984). The failure of either morphine or naloxone to inhibit binding of [^{3}H]leu-

enkephalin to Jurkat cells clearly distinguished the T-lymphocyte receptors from those in the CNS (Ausiello and Roda, 1984). The inhibition of binding of [^{3}H]leu-enkephalin achieved by pretreatment of Jurkat cells with phospholipase A_2 and proteinase K, but not with tunicamycin, suggested that this receptor is a proteolipid with no functionally important carbohydrate (Ausiello and Roda, 1984). That human blood T-lymphocytes have receptors for met-enkephalin was shown initially by its ability to inhibit rosette formation between T-lymphocytes and sheep red blood cells (Wybran *et al.*, 1979). The results of studies of the binding of radiolabeled peptides indicated that the inhibitory effects of met-enkephalin and ACTH on lymphocyte function are mediated by distinct types of receptors with different affinities (Johnson *et al.*, 1982). The receptors on mixed lymphocytes for [^{3}H]met-enkephalin had one K_D of 0.59 n*M*, whereas those for ^{125}I-labeled ACTH exhibited both high and low affinity states with K_D values of 0.1 and 4.8 n*M*, respectively (Johnson *et al.*, 1982), that correspond to those of receptors on adrenal cells (McIhinney and Schulster, 1975). Receptors for β-endorphin have been detected on both blood (Gilman *et al.*, 1982) and cultured lymphocytes (Hazum *et al.*, 1979). The lymphoblastoid line RPMI 6237 expresses receptors for β-endorphin that have a K_D of 3 n*M*, which is in the same range of concentrations required to induce changes in lymphocyte functions (Gilman *et al.*, 1982). As with the lymphocyte receptors for leu-enkephalin, those for β-endorphin differ from those found in the CNS, since binding to the former is not inhibited by naloxone. This suggests that the effect of β-endorphin is not mediated by an opiate receptor of the classical type, but rather by a receptor that interacts with the COOH-terminal portion of β-endorphin (Gilman *et al.*, 1982).

Lymphocytes also have been demonstrated to express specific binding sites for neuropeptides of the PNS that transduce defined immunoregulatory signals (Payan and Goetzl, 1985; O'Dorisio *et al.*, 1985b). Human blood mononuclear leukocytes have approximately 300–500 SOM receptors/cell with a K_D of 0.5 μ*M* (Bhathena *et al.*, 1981). Human and murine lymphocytes also possess receptors for VIP. In the case of T-lymphocytes purified partially from human blood, the specific binding of ^{125}I-labeled VIP, assessed by Scatchard analysis, revealed a single class of receptors with a dissociation constant of 0.47 n*M* and a density of receptors of 1700 sites/cell (Danek *et al.*, 1983; O'Dorisio *et al.*, 1985b). The specific binding of ^{125}I-labeled VIP displayed first-order kinetics and increased linearly with increasing cell concentrations. ^{125}I-labeled VIP bound with similar characteristics to cultured Molt 4B human lymphoblastic cells, where the 15,000 receptors per cell had a K_D of 7 n*M*, that was similar to the affinity of those found on colon, brain, and other target tissues modulated by VIP (Beed *et*

al., 1983). The pharmacologic specificity of the VIP receptor on Molt 4b lymphoblasts was demonstrated by the lack of significant inhibition of binding of ^{125}I-labeled VIP by hormones such as glucagon and secretin. In contrast, the peptide histidine isoleucine (PHI), which shows approximately a 50% homology of amino acid sequence with VIP, effectively competed for specific binding sites (Beed *et al.*, 1983; O'Dorisio *et al.*, 1985b). Structural studies of the VIP receptor on Molt 4b lymphoblasts using disuccinimidyl suberate (DSS), and other bifunctional cross-linking agents, now have shown that ^{125}I-labeled VIP binds specifically to a single membrane-associated protein of M_r 50,000 (Wood and O'Dorisio, 1985).

As for VIP, SP modulates immune function by binding specifically to receptors of T-lymphocytes and other leukocytes, that express immunoregulatory activity (Fig. 6). Experiments using fluorescence detection flow cytometry (FDFC) and fluorescein-labeled SP (SP*) demonstrated the specific binding of SP* to approximately 20–30% of peripheral blood T-lymphocytes (Payan *et al.*, 1984b). Two-color FDFC analysis revealed that SP*-reactive T-lymphocytes were distributed between both the helper–inducer and suppressor–cytotoxic subsets with mean frequencies of 20–30 and 10–20%, respectively (Payan *et al.*, 1984b). The flow cytometric data and the binding of [^{3}H]SP led to the estimation of 35,000 SP receptors per SP*-reactive lymphocyte, with a dissociation constant of 2 n*M*. Competitive binding studies with substituent peptides of SP showed that the carboxy-terminal sequence -Gly-Leu-Met-NH_2 was the principal substituent determining binding (Payan *et al.*, 1984c). In addition, no significant binding of [^{3}H]SP could be demonstrated to peripheral blood B-lymphocytes, monocytes, or platelets, nor to cultured Hut-78 cutaneous lymphoma T cells, Jurkat cells, Molt 4 lymphoblasts, and vitamin D_3-differentiated HL-60 and U-937 monocyte-like cells. The cultured lymphoblastoid cells of the line IM-9 have high affinity binding sites for [^{3}H]SP that belong to a single class of 20,000–25,000 receptors/cell with a K_D of 0.65 n*M* (Payan *et al.*, 1984c). Recent studies have revealed the retention of specific binding of ^{125}I-labeled SP to IM-9 lymphoblast membranes, and to membranes solubilized in CHAPS at a detergent-to-protein ratio of 1.0. The binding kinetics and equilibrium constants for the solubilized membranes were similar to those demonstrated for intact IM-9 cells. Affinity cross-linking of ^{125}I-labeled SP to intact IM-9 cells and membranes by DSS led to recovery of a specifically labeled membrane protein of M_r 58,000 by SDS–PAGE under reducing conditions. Competitive effects of substituent peptides of SP on the binding and cross-linking of ^{125}I-labeled SP to solubilized membranes showed that the SP receptor recognizes the carboxy-terminal domain of SP (Payan *et al.*, 1986).

VII. Thymic Factors

The possibility of bidirectional communication between the thymus and neuroendocrine system was suggested first in relation to the pituitary–adrenal axis. The original anatomical evidence consisted of the demonstration that bilateral adrenalectomy led to an increase in size of the thymus gland (Jaffe, 1924; Marine *et al.*, 1924) and that removal of the thymus elicited enlargement of the adrenal glands in rats (Comsa, 1957). The physiological importance of such observations was supported by the rapid responsiveness of thymic mass to alterations in plasma concentrations of corticosteroids (Akana *et al.*, 1985). The biochemical basis for the regulation by the thymus of immune and endocrine functions has been elucidated recently by the characterization of several distinct polypeptides identified initially in thymic extracts. The thymus-derived polypeptides with immunological activity include thymosin α_1, α_{11}, β_4, β_7, and β_{8-10}, the prothymosin α and parathymosin α precursors of the thymosins, thymopoietins I and II, the pentapeptide fragment of thymopoietin I termed TP5, and thymulin, that previously was designated thymique factor serique (TFS). The source, structural properties, and effects on immunity of the thymic polypeptides have been described comprehensively in recent reviews (Audhya *et al.*, 1984; Dardenne *et al.*, 1984; Goldstein, 1984; Oates and Goldstein, 1984).

The reliance on unpurified and unstandardized thymic extracts, the wide distribution of thymic peptides in antigen-presenting cells of many tissues, the complexity of the immunoassays, and the lack of demonstration of target cellular specificity have complicated systematic definition and interpretation of the effects of thymus-derived peptides. Nonetheless, it is clear that some are potent stimuli of T-lymphocyte differentiation, maturation, and function, including the production of interleukins and interferon. The principal subject of this section is the similarity between thymus-derived peptides and classical neuropeptides, in relation to regulation of adrenal and gonadal function. Several aspects of the generation of thymic peptides are intriguingly analogous to those of neuropeptides. One example is the derivation from the highly acidic 113 amino acid precursor prothymosin α of thymosins α_1 and α_{11}, and possibly of other peptides by cleavage of lysyl-lysine or lysyl-arginine bonds, utilizing a mechanism characteristic of the liberation of neuropeptides from precursor proteins (Haritos *et al.*, 1985). However, the current evidence for the similarity of thymic peptides and neuropeptides rests largely on the results of functional studies.

In the earliest studies, the endocrine deficiencies that followed thymectomy of humans and rats were treated with various thymic extracts and thymus-derived peptides (Anderson, 1932; Deschaux *et al.*, 1979). Plasma concentrations of pituitary, adrenal, and gonadal hormones were normal in

TABLE II
NEUROENDOCRINE EFFECTS OF THYMIC PEPTIDES

Extract or peptide	Model	Effect
Thymic extract[a]	Rats, intraperitoneal injection	Elevation of plasma ACTH and corticosterone
Thymic extract[a]	Monkeys, intravenous injection	Elevation of plasma ACTH, cortisol, and β-endorphin
Thymic extract,[a] thymosin β_4	Rat pituitary tissue	Stimulation of secretion of LH
Thymic extract[a]	Cultured rat anterior pituitary cells	Stimulation of secretion of ACTH
Thymosin α_1	Mice, intracerebral injection	Elevation of plasma concentration of corticosterone, but not LH
Thymosin β_4	Mice, intracerebral injection	Elevation of plasma concentration of LH, but not corticosterone

[a] Thymosin fraction 5 (Oates and Goldstein, 1984).

the treated subjects, as contrasted with deficiencies detected in those not treated. More recently, thymic extracts and, in one instance, thymosin β_4 peptide were shown to elicit release of LH from rat pituitary cells *in vitro* (Rebar *et al.*, 1981) (Table II). In addition, the thymic extracts evoked release of ACTH from rat and monkey pituitary *in vitro* and *in vivo*, without evoking the release of corticoids from adrenal cells (Healy *et al.*, 1983; Vahouny *et al.*, 1983; McGillis *et al.*, 1983, 1985; Oates and Goldstein, 1984; Hall *et al.*, 1985) (Table II). The finding of thymosin α_1 in areas of the hypothalamus critical to pituitary function (Palaszynski *et al.*, 1983), and the observation that the serum concentration varies in a diurnal rhythm opposite to glucocorticosteroids (McGillis *et al.*, 1983) further support the possibility of a role in physiological control of endocrine functions. Nevertheless, much additional work will be required to confirm any role of thymus-derived peptides in the regulation of neuroendocrine functions.

VIII. Neuropeptides and Neuropeptide-Like Factors Endogenous to Cells of the Immune System

The search for immunological mediators capable of regulating neuroendocrine functions (Table III) continues to lead to the discovery of factors native to leukocytes, that are identical or similar to neuropeptides and peptide hormones (Fig. 3). Viral infection, bacterial lipopolysaccharide (LPS), and tumor cells were observed to stimulate human blood leukocytes and mouse spleen cells to produce ACTH and endorphins (Smith and Blalock,

TABLE III
NEUROENDOCRINE MEDIATORS DERIVED FROM CELLS OF THE IMMUNE SYSTEM

Neuroendocrine mediator	Cell source	Criteria for identification
ACTH	Lymphocytes, monocytes	RIA, HPLC, bioassays
CRF-like activity	Lymphocytes	Bioassay
β-Endorphin	Lymphocytes, monocytes	RIA, receptor assay, bioassay, HPLC
TSH	Lymphocytes	RIA
VIP	PMN, mast cells	RIA
SP	Basophils, mast cells, monocytes	HPLC, RIA
SOMs	Basophils, mast cells, monocytes	HPLC, RIA

1981; Blalock *et al.*, 1985). The ACTH and β-endorphin recovered from mononuclear leukocytes of both species were identical to those of the neuroendocrine system by physical, antigenic, and functional criteria (Smith *et al.*, 1982, 1985; Lolait *et al.*, 1984). Analyses of specific cell sources revealed the capacities of both monocytes–macrophages and T-lymphocytes to generate ACTH and β-endorphin (Blalock *et al.*, 1985), but the relative contributions were determined by the cellular preference of the stimulus. Newcastle disease virus elicited the production of neuropeptides by lymphocytes and monocytes, while LPS was more selective for the lymphocytes, especially B-lymphocytes (Smith and Blalock, 1981; Blalock *et al.*, 1985). Although identified by less stringent criteria, a corticotropin releasing factor (CRF)-like activity (Besedovsky *et al.*, 1981, 1985; Bindon *et al.*, 1983; Vahouny *et al.*, 1983), and immunoreactive thyrotropin (TSH), growth hormone, chorionic gonadotropin, follicle-stimulating hormone, and luteinizing hormone all have been detected as products of lymphocytes from rodents and/or humans (Smith *et al.*, 1983; Blalock *et al.*, 1985). The physiological pathways of regulation of secretion of neuropeptides by lymphocytes were documented by the finding that CRF initiated and synthetic glucocorticoids suppressed the appearance of lymphocyte-derived ACTH and endorphins (Blalock *et al.*, 1985; Smith *et al.*, 1986). Thus, peptides identical to those derived from the hypothalamic–pituitary axis are secreted by cells of the immune system and exert potent neuroendocrine activities.

The finding that extracts of PMN leukocytes and mast cells contain immunoreactive VIP (Cutz *et al.*, 1978; O'Dorisio *et al.*, 1980) prompted more detailed investigations of the neuropeptide content of cells participating in immediate and subacute immunologic responses. Basophils, mast cells, and

mononuclear phagocytes of several species, but not erythrocytes, platelets, PMN leukocytes, or lymphocytes have substantial quantities of extractable somatostatin (SOM) and less of SP (Goetzl *et al.*, 1986). The SOM immunoreactivity of rat basophilic leukemia cells (RBL) resembled in size and antigenicity the prepro-SOM, SOM 28, and SOM 14 of neuroendocrine tissues (Goetzl *et al.*, 1985b). However, the "RBL-SOM 28" and "RBL-SOM 14" were resolved completely from synthetic SOM 28 and SOM 14, respectively, by reverse-phase HPLC and had clearly different amino acid compositions (Goetzl *et al.*, 1985b). The "RBL-SOM 28" has 34–35 amino acids, rather than 28, but shares both a methionine and a disulfide bridge with SOM 28. In contrast, "RBL-SOM 28" lacks the tryptophan and phenylalanine of SOM 28, but has tyrosine, valine, leucine, and isoleucine that are not found in SOM 28 (Goetzl *et al.*, 1985b). Although most of the molecular properties and genetic determinants of immunologically derived neuromediators remain to be elucidated, it is clear already that some will be identical to the neuroendocrine peptides and others will share only selected structural and functional characteristics.

IX. Possible Immunopathogenetic Roles of Neuropeptides

The evidence supporting involvement of neuropeptides in a variety of diseases associated with abnormalities of one or more components of immunity (Table IV) has been derived from studies of animal models of disease and, in a few instances, of humans. Increased concentrations of neuropeptides in fluids and tissues of affected systems, relative to those without disease, and the capacity of neuropeptide antagonists to prevent or blunt the expression of aspects of an immunopathological response have constituted the usual proof of involvement.

TABLE IV
PATHOGENETIC INVOLVEMENT OF NEUROPEPTIDES IN DISEASES

Peptide	Disease	Species	Site
SP	Arthritis	Rat	Regional nerves
SP	Asthma	Guinea pig	Bronchial tissue
SP	Allergic rhinitis	Humans	Nasal secretions
SP	Urticaria	Humans	Cutaneous tissue
SP	Hepatic coma	Humans	Plasma
SP	Chronic arachnoiditis	Humans	Lumbar spine CSF
VIP	Cystic fibrosis	Humans	Nerves of eccrine sweat glands (deficiency)

The similarity between the distribution of primary afferent nerves and the symmetrical and preferentially distal occurrence of experimental arthritis in rats suggested that mediators from the nerves might participate in the development of arthritic lesions (Levine *et al.*, 1984, 1985). This contention was supported by the detection of higher concentrations of SP in regional nerves supplying arthritic rat joints (Lembeck *et al.*, 1981), the lesser severity of arthritis in joints of limbs with a diminished afferent nerve supply or content of neuropeptides in the nerves (Courtwright and Kuzell, 1965; Colpaert *et al.*, 1983), the capacity of intraventricular morphine to suppress arthritis in rats at concentrations that inhibit spinal nociceptive nerve functions (Levine *et al.*, 1985), and the accentuation of arthritis in rats that follows the intraarticular instillation of SP for a few days early in the course of experimental induction (Levine *et al.*, 1984). Comparably detailed analyses of the role of SP in immunologically induced bronchospasm have been completed in guinea pigs and rats. Pretreatment of the animals with specific antagonists of SP reduces significantly the bronchospasm and respiratory mucosal edema elicited by capsaicin, that releases endogenous SP, vagal neurogenic stimulation, or chemical irritation (Lundberg *et al.*, 1983; Lundberg and Saria, 1983).

In the majority of reports, the neuropeptides are implicated in pathogenesis solely by the chemical or immunochemical detection of high concentrations in extracts of lesional tissue or fluids, or by the demonstration that locally administered neuropeptide mimics the features of hypersensitivity (Table IV) (Hosobuchi *et al.*, 1982; Hortnagl *et al.*, 1984; Heinz-Erian *et al.*, 1985). The elicitation of a classical wheal and flare response in human skin by SP, although the flare is histamine dependent, exemplifies the latter approach (Foreman and Jordan, 1983). Other complex issues, such as the opposing effects of different neuropeptides, have not been addressed meaningfully in studies of intact animals or man. For example, VIP opposes the bronchoconstriction evoked by histamine and SP *in vivo* (Morice *et al.*, 1983), but a role in asthma for this and related mechanisms has not been assigned definitively.

X. Future Directions

The complex bidirectional interactions between the immune and nervous systems encompass many aspects of cellular proliferation, differentiation, distribution in organ systems, and function. The projected course of research in neuroimmunology will lead to characterization of the structure and genetic determinants of neuropeptides and receptors for neuropeptides in the nervous, endocrine, and immune systems. The application of this information, in addition to the resultant immunochemical and genetic tools, should

permit the identification of sites of synthesis and localization of the mediators and their receptors in organs of host defense, such as the skin, lungs, and gastrointestinal tract, as well as the immune and nervous systems. It also will be possible to define the roles of local neural factors in the regulation of lymphocyte traffic, regional immunological responses, and hypersensitivity reactions restricted to distinct tissue compartments. A greater understanding of the stereospecificity of receptors for immunological mediators and neuropeptides will provide novel approaches to the design of synthetic molecules capable of acting as selective agonists and antagonists. Such highly specific probes will be employed in studies of normal neuroimmunological functions and neuroimmunological contributions to human diseases.

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