Fatty Acids and Immunity

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I. Introduction

The idea that fatty acids may play a role in immunity is new, and no comprehensive earlier review exists. Parts of the field, however, have been surveyed by other workers. Resch and Ferber (1975) wrote an excellent account of their studies on the role of fatty acids in lymphocyte stimulation, updated in a 1976 German review (Ferber and Resch, 1976). The 1972

volume of this series contained a detailed account by diLuzio of the effects of emulsions of fatty acid esters on the reticuloendothelial system. Recently, Pelus and Strausser (1977) reviewed prostaglandins and immunity. We have written this review in an attempt to gather together these and other threads from the fields of immunology, biochemistry, and nutrition. Our primary aim has been to provide a new perspective rather than to summarize an established field. It is early yet to know whether fatty acid research may finally find a niche in immunology. We expect most of our readers to be lipid biochemists who may be unfamiliar with much of the literature that has appeared in immunological journals. Readers with only an immunological background may find useful the chapter on fatty acids in Gurr and James (1975).

This review will consider its topic from three viewpoints. First we shall examine the role of fatty acids, both as membrane components and prostaglandin precursors, in the biochemistry of the cells of the immune system. Second, we shall describe the effects of altered fatty acid concentrations on the immune response *in vivo* and *in vitro*. Finally, we shall try to show that some of the existing data on the relationship between dietary essential fatty acids and disease can be interpreted, among other ways, in terms of effects on the immune system.

II. Biosynthesis and Uptake of Fatty Acids by Lymphocytes

Like all mammalian cells, lymphocytes contain a variety of fatty acids, some of which, the essential fatty acids, must be supplied to the cells since they are unable to synthesize them. Of the essential fatty acids, linoleic acid and the derivative arachidonic acid are major components of the phospholipids of lymphocyte membranes (see Table I), while α-linolenic and its derivatives are only minor components. Essential fatty acids are polyunsaturated (i.e., having more than one double bond in the carbon chain) and lymphocytes lack the ability to perform the particular desaturation necessary for their synthesis from saturated precursors. Thus, arachidonic acid cannot be synthesized *de novo,* although it can, in most species, be made from linoleic acid by chain elongation (Liljeqvist, 1973). Essential fatty acids must be present in the mammalian diet for health to be maintained. Changes in the proportion of dietary essential fatty acids lead to changes in the fatty acid composition of lymph nodes (Meade *et al.,* 1978) or spleen cells (Tsang *et al.,* 1976).

Lymphocytes can synthesize all their nonessential fatty acids. This is shown by their ability to incorporate appreciable quantities of radioactive label from acetate-1-^{14}C into all fatty acids except linoleic acid (Blomstrand

Table I
ALTERATIONS IN FATTY ACID CONTENT OF PHOSPHATIDYL CHOLINE FROM RABBIT THYMUS CELLS FOLLOWING STIMULATION BY CONCANAVALIN A [a,b]

	Moles %						Polyunsaturated/ saturated fatty acids
	16:0	18:0	18:1	18:2	20:4	22:6	
Position 1							
Control	50.6	17.9	22.5	4.9	—	—	0.072
Con A	58.1	16.0	20.7	5.2	—	—	0.070
Position 2							
Control	47.4	3.2	20.0	19.4	7.8	—	0.538
Con A	39.0	1.6	21.7	20.3	17.2	—	0.924

[a] The cells were cultured for 4 hours in Eagle's medium with 5 μg concanavalin A/ml.
[b] From Ferber and Resch (1976).

and Liljeqvist,1972; Liljeqvist, 1973). When a lymphocyte is stimulated, either specifically by antigen or nonspecifically by plant lectins such as phytohaemagglutinin or concanavalin A, a variety of metabolic changes occur which may result in cell division, antibody production, or a number of other responses. In unstimulated lymphocytes very little label is incorporated from acetate-1-^{14}C into palmitic or myristic acids, suggesting that incorporation of label is achieved chiefly by chain elongation. However, in stimulated lymphocytes cultured in lipid-free media, appreciable radioactivity appears in palmitic acid, most of it in carbon atoms other than the terminal carboxyl, implying *de novo* synthesis.

Studies of *in vitro* stimulation often use plant lectins, which react with a higher proportion of lymphocytes than most antigens. Liljeqvist (1973) and Resch and Ferber (1972) agree in finding incorporation of label from acetate-1-^{14}C into fatty acids to be increased following stimulation of lymphocytes by phytohaemagglutinin. They disagree, however, on the fate of this label. Liljeqvist, using human thoracic duct lymphocytes, found ^{14}C in all lipid fractions, but less in phospholipid. Resch and Ferber, using rabbit mesenteric lymph node cells, found preferential incorporation into the phospholipid fraction. Any of a number of differences in experimental design might explain this discrepancy. One point we would like to mention is that incorporation of radioactive label depends not only on rates of biosynthesis but also on the size of the pool of "cold" fatty acids available to compete with fatty acids formed from acetate-1-^{14}C for enzymes incorporating fatty acids into phospholipid. An increase in the size of the cold pool will decrease the incorporation of labeled fatty acids into phospho-

lipid. Liljeqvist incubated lymphocytes for 18 hours, adding labeled acetate 6 hours before the end of incubation, while Resch and Ferber had acetate-1-^{14}C present throughout. An early phytohaemagglutinin-stimulated increase in the size of the cold pool might explain the discrepancy.

III. Fatty Acids as Lymphocyte Components

A. Lipid Composition of Lymphocyte Membrane

The lymphocyte is, like other cells, surrounded by a membrane consisting chiefly of proteins and phospholipids such as phosphatidylethanolamine and phosphatidylcholine. Table I shows the main fatty acids at position 1 and position 2 of phosphatidylcholine from rabbit thymus lymphocytes. In resting lymphocytes (the "control" of Table I), the ratio of polyunsaturated to saturated fatty acids in position 2 is lower than in phosphatidylcholine from rat liver (a widely used phospholipid source). This is true for phosphatidylcholine from lymphocytes of a number of species (Ferber *et al.*, 1975). Modern concepts of cell membranes see them not as rigid, but in a dynamic state in which parts of the membrane lipid are constantly passing from an ordered to a fluid state (Singer and Nicolson, 1972). Although many factors contribute to membrane fluidity, membranes having a lower ratio of polyunsaturated to saturated fatty acids are generally less fluid than those having a higher ratio (Overath and Träuble, 1973).

Of the proteins in the lymphocyte membrane, antigen receptors and histocompatibility antigens are of particular immunological importance. The latter, present also on many cell types other than the lymphocyte, determine whether a cell will elicit a specific immune reaction. Although there is, as yet, no evidence for modulation of the biological activity of antigen receptors or histocompatibility antigens by their lipid environment, in experiments on a variety of cell types it has been shown that the enzymic or receptor activity of membrane proteins can be modulated by the composition, including fatty acid composition, of surrounding lipid (see Table II).

According to the "fluid mosaic model" of Singer and Nicolson, proteins are distributed randomly, so that unless the ratio of protein/lipid is very high, long-range cooperative effects between proteins must be transmitted through the lipid phase. Immunologically important long-range cooperative effects might include those between two or more antigen receptors, between antigen receptors and membrane enzymes, and between viruses and histocompatibility antigens. A hint that one such interaction might be through the fluid phase is the finding of Pfizenmaier *et al.* (1977) that *in vitro* killing

Table II
EXAMPLES OF MODULATION OF MEMBRANE PROTEIN ACTIVITY BY FATTY ACID COMPOSITION OF SURROUNDING LIPIDS

System	Membrane protein activity	Reference
Escherichia coli	β-Galactoside transport system	Schairer and Overath (1969)
3T3 cells (a mouse fibroblast line)	Agglutinability of lectin receptors	Horwitz *et al.* (1974)
Rat erythrocyte	Allosteric activation or inhibition of ATPase, acetylcholinesterase, and *p*-nitrophenylphosphatase	Farias *et al.* (1975)
Mouse LM (fibroblast) cells	Adenylate cyclase activity	Engelhard *et al.* (1976)
Tetrahymena periformis	Fatty acid desaturase activity	Kasai *et al.* (1976)

of cells treated with inactivated Sendai virus cannot occur when the target cells are held at 4°C prior to exposure to attacker lymphocytes. Such killing involves target cell histocompatability antigens, since it can only occur when such cells have histocompatibility antigens in common with the cells used to prime the attacker lymphocytes. One interpretation of these results is that ordering or "freezing" fluid membrane at 4°C interferes with virus-histocompatibility antigen interaction. Figure 1 speculates how a change in the lipid phase might interact with a histocompatibility protein to cause a cell to be sensitive to immunological attack.

B. CHANGES IN FATTY ACID COMPOSITION OF LYMPHOCYTE MEMBRANES FOLLOWING STIMULATION

Membrane phospholipid fatty acid composition alters when lymphocytes are stimulated, either by antigen or by mitogens such as phytohaemagglutinin or concanavalin A (Table I). Most marked is an increase in arachidonic acid at position 2 (Ferber *et al.*, 1975). There is a rapid turnover of the fatty acids in the phosphatidylcholine of phytohaemagglutinin stimulated lymphocytes (sufficient to replace total cell phosphatidylcholine fatty acids within less than 10 hours). The alteration in fatty acid composition of this phospholipid after stimulation is largely a result of such turnover, rather than of *de novo* synthesis of new phospholipid molecules. Evidence for this comes from studies in which incorporation of oleic acid-1-^{14}C into phosphatidylcholine was used as a measure of fatty acid turnover. Simultaneous measurement of the incorporation of [^{14}C]choline could be

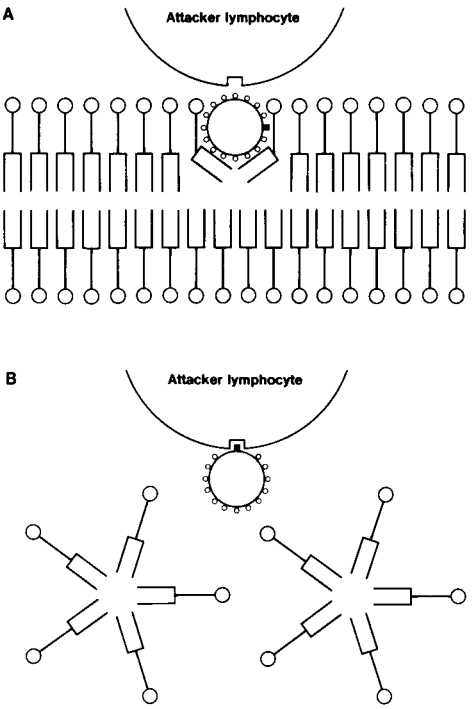


FIG. 1. How alterations in binding energies to lipid might cause a membrane protein to expose a certain antigenic determinant. In (A) the antigenic determinant is masked because attraction between hydrophobic parts of the protein molecule and membrane lipid places the antigenic determinant where it cannot be "seen" by a lymphocyte. In (B) as a result of, for example, interaction of the membrane with a virus, the phospholipid molecules are forced to adopt a new arrangement in which they can less easily interact with the protein molecule, which therefore alters its conformation to expose the determinant (hypothesis only).

used to assess *de novo* phosphatidylcholine biosynthesis, because there is only one pathway for putting a choline residue in the phosphatidylcholine molecule. That is from choline via cytidine diphosphatecholine (see Fig. 2). Resch and Ferber (1972) found that, under similar conditions, [^{14}C]-oleate incorporation into phosphatidylcholine could be 100-fold higher than [^{14}C]choline incorporation.

Ferber and Resch (1973) have described a plasma–membrane associated enzyme, catalysing incorporation of coenzyme A derivatives of fatty acids into phospholipid, and activated by phytohaemagglutinin or concanavalin A (Fig. 3). Affinity of this enzyme is greater for more highly unsaturated fatty acids, particularly arachidonic acid (Table III). Such a transferase enzyme can explain the preferential incorporation of arachidonate into the phospholipids of activated lymphocytes. It does not, however, explain why the arachidonic acid is preferentially incorporated into the 2 position since

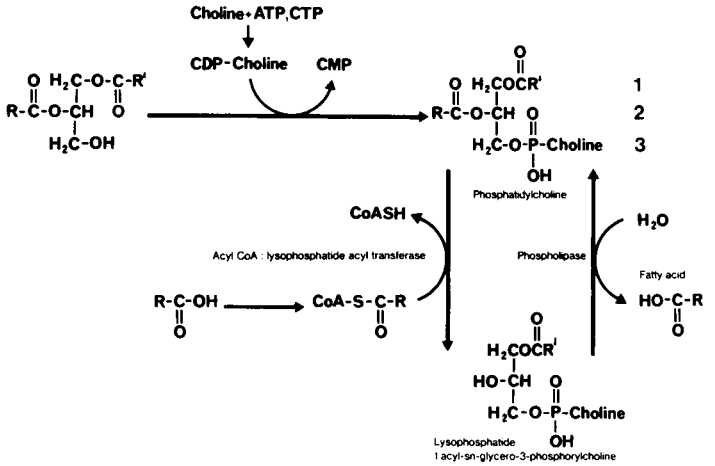


FIG. 2. Metabolism of phosphatidylcholine. The numbers 1, 2, and 3 identify the carbon atoms in the glyceryl backbone of the molecule.

the enzyme has similar affinity for lysophosphatide with either the 1 or 2 positions available for acylation by fatty acid.

Many enzyme activities are altered when lymphocytes are stimulated. There is evidence, however, that acyltransferase activation is a direct consequence of mitogen binding. First, neither mitogen binding nor enzyme activation is affected by puromycin, or by low temperature (0°C), suggesting enzyme activation requires neither protein biosynthesis nor energy metabolism. Second, both processes are complete within 30 min at 37°C.

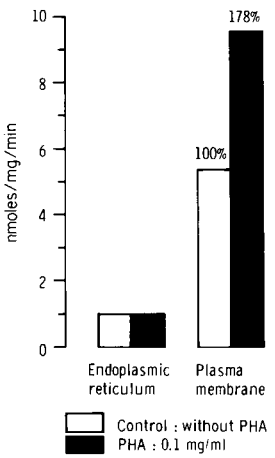


FIG. 3. Oleoyl CoA: 1 acyl-*sn*-glycero-3-phosphorylcholine acyltransferase in plasma membranes and endoplasmic reticulum from rabbit lymphocytes after 1 hour of stimulation with phytohaemagglutinin. From Ferber and Resch (1973).

Table III
SUBSTRATE SPECIFICITY OF MICROSOMAL ACYL CoA:1-ACYL-*sn*-GLYCERO-3-PHOSPHORYLCHOLINE ACYLTRANSFERASE OF CONCANAVALIN A-STIMULATED THYMUS CELLS [a]

	Oleoyl-CoA (18:1)		Arachidonyl-CoA (20:4)	
	V_{max} [b]	K_m M	V_{max}	K_m M
Control	3.9	1.0×10^{-5}	7.6	6.4×10^{-7}
Con A	7.9	1.2×10^{-5}	31.7	8.5×10^{-7}

[a] From Ferber and Resch (1976).
[b] V_{max} is given in nanomoles per milligram protein per minute.

Third, if within 30 min of first exposure binding of concanavalin A to its receptor is reversed by α-methylmannoside, a competitive ligand, the enzyme activation is also interfered with. Stepwise elution of membrane fractions from concanavalin A-sepharose using α-methylmannoside gives greatest specific activity of acyltransferase in the fraction most strongly able to bind concanavalin A (Ferber and Resch, 1976). There *are* differences in the kinetics of concanavalin A binding and acyltransferase activation. With increasing concanavalin A concentration, concanavalin binding increases with the usual hyperbolic kinetics associated with a simple, saturatable receptor. By contrast, activation of the transferase enzyme follows sigmoid kinetics. These differences do not, however, exclude the possibility that acyltransferase activation is a direct consequence of mitogen binding. The differences could, for example, be explained if, for transferase activation to occur, it was necessary not only for one concanavalin A receptor site to be occupied, but also for an interaction to occur between it and other occupied receptor sites. Such might be the case if aggregation of concanavalin A receptors was required for enzyme activation (Ferber and Resch, 1976).

C. A ROLE FOR FATTY ACIDS IN LYMPHOCYTE ACTIVATION

One of the fundamental questions in immunology is how the occupation of a receptor by antigen causes a lymphocyte to divide, produce antibody, or engage in any of the multitude of other specific responses to antigen. This problem is important not only for immunology, but also for cell differentiation generally, for the ease with which the lymphocyte can be stimulated to differentiate by a simple experimental stimulus makes it a useful model. The problem is complicated by the existence of two major subclasses of lymphocytes, T and B cells. B cells produce antibody when stimulated by antigen. Sometimes the antigen alone is not sufficient stimulus

and antibody will not be produced until there is "help" from a T cell, possibly via a soluble T cell product.

An important aspect of the problem is how a receptor–ligand interaction on the outer surface of the cell membrane alters activity of a wide variety of enzymes within the cell. Attention therefore centers on changes in the membrane, early after lymphocyte stimulation, which alter the internal environment in such a way as to affect many enzyme activities. One such change is an increased permeability to ions, particularly Ca^{2+} (Quastel and Kaplan, 1971; Whitney and Sutherland, 1972). Another is altered cyclic nucleotide levels (Smith *et al.*, 1971a; Hadden *et al.*, 1972). Resch and Ferber (1975) have suggested that changes in membrane fatty acid composition occurring shortly after lymphocyte stimulation might be the primary "signal" triggering the lymphocyte. Their hypothesis does not necessarily exclude a role either for solute fluxes or cyclic nucleotides, for both of these can be affected by the fatty acid composition of a membrane (Demel *et al.*, 1972; Engelhard *et al.*, 1976). Although most of their evidence comes from studies on lymphocyte stimulation by phytohaemagglutinin or concanavalin A, both of which only stimulate T cells, they have found that fatty acid turnover, as measured by incorporation of oleic acid-1-^{14}C into phosphatidylcholine, can also be stimulated by anti-immunoglobulin serum, a B cell mitogen (Resch and Ferber, 1972). Any theory involving fatty acid changes in B lymphocyte activation must also take into account the requirement of B cells for T cell help.

Resch and Ferber argue that the increased proportion of polyunsaturated fatty acids in lymphocyte phospholipids obtained early after stimulation is a sufficient explanation for the increased membrane fluidity which they (Ferber *et al.*, 1974) and others (Barnett *et al.*, 1974) report. As discussed earlier, membrane enzyme function and permeability can be altered by alterations in the lipid layer, and Resch and Ferber see the altered state of the lipid layer as being the underlying change which leads to those other changes that culminate in lymphocyte differentiation.

The technique Ferber *et al.* used to measure fluidity was the temperature dependence of the fluorescence polarization of perylene. If a fluorescent probe is excited by polarized light, it will reemit partially depolarized light, the degree of depolarization depending principally on the fluorescent lifetime of the probe and the fluidity of the probe environment. If the environment is very fluid, or if the fluorescent lifetime is long, the light will be completely depolarized. If it is assumed that the fluorescent lifetime is not affected by the membrane, then the temperature at which depolarization is complete gives an indication of the fluidity of the membrane. Using electron spin resonance, Barnett *et al.* (1974) also observed an increase in the fluidity of the membrane environment of their probe 6-(4′4′-dimethyloxazolidinyl-*N*-oxyl)heptadecanoate.

The principal problem about using probes in membranes is that they only provide information on their immediate environment, and it is often far from certain where this is (a discussion of this problem appears in the review by Nicolau *et al.*, 1977). The problem is particularly acute when whole cells are used, as in the study by Toyoshima and Osawa (1976). These workers followed changes in lymphocyte membrane fluidity using 1,6-diphenyl-1,3,5-hexatriene as their fluorescent probe. They found that the temporary increase in membrane fluidity following lectin binding was not accompanied by a particular incorporation of labeled polyunsaturated fatty acids. Sixty minutes after lectin binding, incorporation of [^{3}H]arachidonic acid [^{14}C]-1-linoleic acid was increased, but by this time membrane fluidity had virtually returned to normal. If the fluorescent probe and fatty acid incorporation measured were truly both in the lymphocyte plasma membrane, these results argue against an explanation of fluidity changes in terms of alterations in membrane fatty acid composition.

The concept that it is changes in the phospholipids which underlie lymphocyte triggering has intriguing implications for the control of lymphocyte function (Barton and Diener, 1975). As mentioned earlier, certain T cells appear to modulate the activity of B cells and, recent research suggests, of other T cell subpopulations, in part at least by means of soluble products. A triggering mechanism involving lipids implies that a molecule capable of interacting with the lipid layer would be particularly suitable as a modulator. One of the substances produced by T cells, and capable of specifically helping B cells to produce antibody, has been shown by Taussig and Munro (1974) to react with antiserum directed against certain histocompatibility antigens. Histocompatibility antigens exist on a very hydrophobic collection of proteins, which readily associate with lipids. Thus, one particular histocompatability antigen (HLA 7), when solubilized, occurs in the β-lipoprotein fraction of human serum (Charlton and Zmijewski, 1970). Normally, however, histocompatibility antigens are firmly anchored by hydrophobic forces within the lipid layer (Nathenson and Cullen, 1974). The presence of histocompatibility antigens as well as an antigen binding site on the soluble factor described by Taussig and Munro implies that such a factor might readily interact with the lipid layer.

IV. Role of Free Fatty Acids

Increased turnover of phospholipid fatty acids following lymphocyte stimulation requires not only a transferase to incorporate new fatty acids (as fatty acyl CoA's) but also a means of generating lysophosphatides and free fatty acids as substrates for this enzyme. Lysophosphatides can only

be formed by cleavage of a fatty acid from phospholipid and radioisotope incorporation data suggest most of the free fatty acids incorporated into phospholipid are also derived in this way (Resch *et al.*, 1971). However, lymphocytes are not particularly rich in the cleaving enzymes and changes in phospholipase A activity on lymphocyte activation are only modest. Resch *et al.* (1971), using red cells containing labeled lecithin as substrate, found only a 30% increase in membrane associated phospholipase activity following phytohaemagglutinin stimulation of rabbit lymphocytes. In studies in which fatty acid release from [1-(1-^{14}C)]palmitoyl-*sn*-glycerol-3-phosphorylcholine was measured, no increased lysophospholipase activity could be observed at all (Ferber and Resch, 1973; Resch *et al.*, 1971). Possibly the lymphocyte membrane enzyme is not the only means of cleaving phospholipids. Is there another phospholipase involved? *In vitro* and *in vivo* stimulated lymphocytes are closely associated with macrophages or similar cells (McFarland *et al.*, 1966). Macrophages are required for optimal stimulation of T cells (Alter and Bach, 1970) as well as for the B cell response to T cell dependent antigens (Feldmann and Palmer, 1971). Macrophages have much more phospholipase activity than lymphocytes, and when stimulated can release this enzyme (Munder *et al.*, 1969). We speculate the macrophage may be a possible source of the enzyme generating lysophosphatides in the lymphocyte membrane, and phospholipase as one possible mediator of lymphocyte–macrophage interaction.

As well as lysophosphatide, the "turnover" biosynthesis of new phospholipid in stimulated lymphocytes requires a source of free fatty acids. There are marked differences between control and *in vivo Bacillus calmette guerin*-(BCG) stimulated rabbit lymphocytes with respect to the composition of their free fatty acid pool. In particular, arachidonic acid, which makes up 11% of the control free fatty acid pool, is not detectable in the free fatty acids of BCG stimulated lymphocytes (Ferber *et al.*, 1975). Nevertheless, the pool of free fatty acids utilized for phospholipid biosynthesis does not appear to exchange freely with the pool of free fatty acids within the lymphocyte. After phytohaemagglutinin or concanavalin A stimulation of rabbit lymph node cells in the presence of [1-^{14}C]acetate, ^{14}C incorporation into phosphatidylcholine is enhanced 30-fold, but into free fatty acids only threefold (Resch and Ferber, 1972). Cyong and Okada (1976) have demonstrated histochemically greatly increased quantities of free fatty acids between and on the surface of stimulated lymph node cells. Possibly this pool of fatty acids is also available to the transferase enzyme. To demonstrate *in vitro* that stimulated lymphocytes release free fatty acids, Cyong and Okada (1976) cultured a mixture of primed lymphocytes with other lymphocytes or tumor cells of a different histocompatability type. A soluble antigen, purified protein derivative of tuberculin (PPD), was also

effective. In all these situations, T cells are of the predominant stimulated cell type.

Free fatty acids have a marked lytic action on cells of all types. This has led to the suggestion that release of free fatty acids during the immune response may be one of the mechanisms by which lymphocytes can kill other cells, including tumor cells (Kigoshi and Ito, 1973; Okada and Cyong, 1975). Lymphocytes, compared with other cell types, contain higher concentrations of free fatty acids. Resch and Ferber (1975) report free fatty acids to be about 9% of the total lipids of calf thymus lymphocytes. Guinea pig lymph nodes stimulated with complete Freund's adjuvant contain as much as 18% free fatty acids (Kigoshi and Ito, 1973). By contrast guinea pig liver, heart, lung, or kidney tissue contain only 2–3% free fatty acids. The figures for stimulated and unstimulated guinea pig spleen are 14% and 17%, respectively.

The measurement of free fatty acid levels in lymphoid tissues poses special problems, because endogenous phospholipase may be released when tissues are homogenized. Interest in a possible cytolytic role for free fatty acids was first aroused by attempts to identify a factor, present in extracts of normal lymph nodes and capable of killing a wide variety of cells, including tumor cell lines (Okudaira *et al.*, 1970). This factor turned out to be free fatty acids generated during the extraction procedure. If the lymph nodes were first heat treated to destroy any lipases, then no killing activity could be demonstrated. However, later experiments showed that, even though no cytolytic activity was associated with lipid extracts of unsensitized cells, an ability to lyse red cells was present in a hexane extract of sensitized lymph node cells, incubated for 1 hour with the specific tumor line used for immunization. The control in which immune cells were incubated with a tumor cell line other than that against which they were immunized, was negative. The extracts were prepared in such a way that both intracellular and extracellular lipids were assayed (Okada and Cyong, 1975).

Two important mechanisms by which lymphoid cells kill other cells *in vitro* are direct action of a subpopulation of specifically immune T cells, and a mechanism involving specific antibody and a subpopulation of lymphoid cells termed *K cells*. During T cell killing, there is no killing of "bystander" cells bearing antigens other than those against which the T cells are sensitized (Berke *et al.*, 1972). Similarly during K cell killing, there is no killing of bystanders not bearing antigens against which the specific antibody is directed (C. J. Sanderson, personal communication). These results make it unlikely that fatty acids, freely released into the medium surrounding tumor cell "targets," can have a role in tumor cell killing, at least by the mechanisms mentioned. They do not, however, ex-

clude a transfer of free fatty acids (poorly soluble in aqueous media) during a contact between tumor cell and lymphocyte. Such contacts are essential for T cell killing, although cell death can occur after detachment of killer lymphocytes (Sanderson and Taylor, 1975).

The possible involvement of free fatty acids during the early events following lymphocyte stimulation and in the cytotoxic action of lymphocytes, together with recent evidence that prostaglandin biosynthesis can be affected by the availability of free fatty acid substrate (see next section) has led us to consider a possible regulatory function for free fatty acids either within the cell or in the local lymphatic microenvironment. Injected fatty acids, particularly arachidonic acid, can produce (with increasing total dosage) stimulation of lymphoid cell division, immune unresponsiveness, or lymphocytolysis (Meade and Mertin, 1976). The ability to produce these effects would be a suitable property for an immunoregulatory substance. Lymphocytes possess receptors for the major hormones involved in the regulation of fatty acid metabolism—insulin (Krug *et al.*, 1972) and corticosteroids (Turnell and Burton, 1975). They also have β-adrenergic receptors (Williams *et al.*, 1976). The physiological role of such hormone receptors is largely unknown, although corticosteroids have long been used clinically as immunosuppressants. Steroids are known both to stimulate and to inhibit phospholipase activity (Blackwell *et al.*, 1977). Could they, and perhaps other hormones, interact with the immune system by effects on fatty acid release?

Turnell and colleagues have suggested that glucocorticoid induced lymphocytolysis is mediated through a release of free fatty acids within the cell (Turnell *et al.*, 1973; Turnell and Burton, 1974, 1975). The evidence produced in support of this hypothesis is as follows:

1. The steroid dexamethasone raises the level of free fatty acids in thymic lymphocytes or in cells of a corticosteroid-sensitive mouse lymphosarcoma P1798S, but not in the steroid-resistant subline P1798R.
2. *In vitro,* relatively low concentrations of long-chain free fatty acids cause changes in P1798S cells or thymocytes similar to those produced by steroids, viz., disintegration of the nuclear membrane and ultimately karyolysis. The time sequences of the steroid and fatty acid induced effects are similar. However, the steroid-resistant P1798R subline requires 10-fold higher concentrations of added free fatty acids to show such effects.
3. "Naked" nuclei of either resistant or sensitive sublines are insensitive to doses of steroids similar to those which damage whole cells. Hence the basis of resistance in the P1798R subline lies outside the nucleus, although this is the first organelle to be damaged. However, nuclei from either subline are sensitive to free fatty acids.

4. Resistant subline cells, forced to accumulate free fatty acids by having their free fatty acid metabolism blocked by citral undergo lymphocytolysis. Turnell and colleagues visualize the source of free fatty acids being intracellular triglycerides, the level of which is higher in sensitive than resistant P1798 sublines.

Essential fatty acid deficiency potentiates the thymolytic activity of glucocorticoids in male mice, but the mechanism of this is unknown (C. J. Meade and J. Sheena, unpublished).

A second way in which lymphocyte fatty acid concentration might be regulated is via their interrelationship with cholesterol concentrations. Lymphoid cells have, compared with lung, liver, heart or kidney tissue a high level of sterol esters (Kigoshi and Ito, 1973). There is a good correlation between concentrations of free cholesterol and free fatty acids within lymphoid cells (Kigoshi *et al.*, 1976). This might indicate (a) cholesterol esters are the major source of lymphoid cell free fatty acids (but cholesterol esters generally are less liable to hydrolysis than triglycerides), (b) free fatty acids control cholesterol levels, or (c) cholesterol levels control free fatty acid concentrations. Biosynthesis and uptake of cholesterol by lymphocytes are in turn regulated by a low density lipoprotein for which lymphocytes can develop a specific receptor (Ho *et al.*, 1976, 1977). We still need to know (1) the relation of this lipoprotein to the low density lipoprotein which suppresses, even at low concentrations, immune function both *in vitro* (Curtiss and Edgington, 1976) and *in vivo* (Curtiss *et al.*, 1977); (2) whether the ability of dietary linoleate to lower serum levels of some lipoproteins (Nichaman *et al.*, 1967) extends to either the cholesterol binding or immunoregulatory lipoproteins; and (3) whether diets of different linoleate content, which alter plasma cholesterol concentrations (Alfin-Slater *et al.*, 1954), also affect lymphoid tissue cholesterol levels.

A third way in which fatty acids might be involved in the regulation of immune responses is via their release from brown fat. Brown fat deposits are prominent in both very young and hibernating animals, both of which show poor immune responses (Brent, 1958; Sidky and Auerbach, 1968; Sidky *et al.*, 1969; Sidky and Hayward, 1972). Removal of most of the brown fat in neonatal rats enhances T cell responses (e.g., rejection of a thyroid graft, the delayed skin reaction to bovine serum albumin, the severity of an autoimmune response to brain tissue), but only if the thymus is also present (Janković *et al.*, 1975). There is no effect on B cell functions (e.g., production of antibodies against sheep red blood cells or bovine serum albumin). The active factor can be extracted with chloroform (Sidky *et al.*, 1972). It is attractive to relate these results to the depression of thymus weight by arachidonic and other fatty acids (Meade and Mertin,

1976), but in the absence of any data on the effects of removal of brown fat on levels of different fatty acids in serum and lymphoid organs, we consider such an interpretation highly speculative. Other possibilities, e.g., that brown fat acts as a "reservoir" for corticosteroids, must also be considered (Ratsimamanga and Nigeon-Dureuil, 1959).

V. Fatty Acids in Macrophages

The fatty acid profile of macrophages has been little studied despite the importance of this cell type in phagocytosis and killing of microorganisms, and in the modulation of lymphocyte function. Analysis of the fatty acid composition of the macrophage plasma membrane is hampered by the difficulties that are involved in its separation from the relatively large quantities of endoplasmic reticulum characteristic of this cell type. However, the membranes of phagocytic vesicles can be separated. Most of their lipid is derived from the cell membrane. Arachidonic acid constitutes almost 20% of the total phospholipid fatty acid content of macrophages (Mason *et al.*, 1972). The phagocytic vesicles of rabbit alveolar macrophages also contain a high proportion (just less than 15%) of arachidonic acid, but the overall ratio of polyunsaturated to saturated fatty acids is slightly lower than that of the whole cell.

Macrophage membranes undergo profound morphologic changes when exposed to particulate matter, immune complexes, or nonantibody soluble T cell products (lymphokines). Macrophages, like lymphocytes and many other cell types, possess enzyme(s) capable of acylating lysophosphatides. Elsbach and Levy (1968) showed that phagocytosis of inert particles by rabbit alveolar macrophages tripled the formation of phosphatidylcholine or phosphatidylethanolamine from their respective labeled lyso-derivatives. It is not known, however, what part increased acylation of lysophosphatides by fatty acids plays in the phagocytosis process. However, alteration of the fatty acid composition of macrophage membranes by growth in media of different fatty acid compositions does affect phagocytic activity. Schroit *et al.* (1976) reported decreased uptake of ^{125}I-labeled Shigella by murine macrophages grown in medium containing elaidic acid (trans, 18:1) instead of oleic acid (cis, 18:1).

Macrophage lysosomes are rich in lipases, and during phagocytosis hydrogen peroxide is generated. There is evidence that peroxidation of polyunsaturated fatty acids such as arachidonic acid, occurs during phagocytosis. Assay of malondialdehyde, produced by the peroxidation of certain unsaturated fatty acids, is an easy way of quantifying this process. Alveolar macrophages produce malondialdehyde when ingesting polystyrene beads

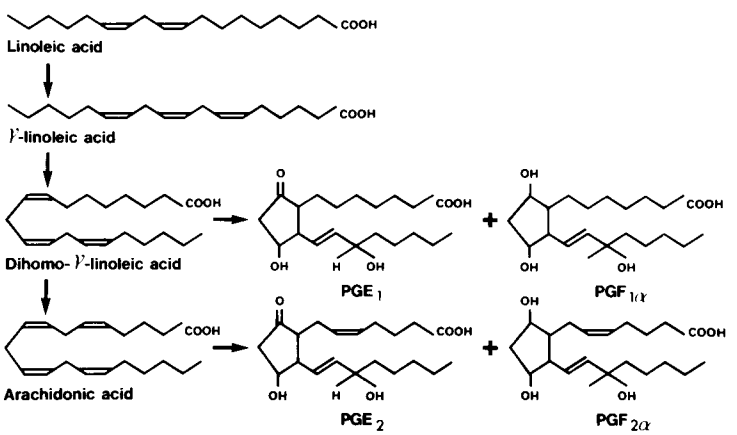
or emulsified paraffin oil (Mason *et al.*, 1972). Aldehydes such as those produced by lipid peroxidation may play a role in bacterial killing (Jacobs *et al.*, 1970).

The possibility that lysosomal lipases may act to release arachidonic acid from macrophage membranes has important implications, for example for the role of macrophages in prostaglandin production. These will be discussed in the next section.

Aluminium hydroxide and arlacel A enhance the breakdown by macrophages of phospholipids to free fatty acids and lysophosphatides, and it has been suggested that such breakdown may underlie the adjuvanticity of these substances (Munder *et al.*, 1969).

VI. Fatty Acids as Prostaglandin Precursors

Some derivatives of linoleic acid, notably di-homo-γ-linolenic acid and arachidonic acid, can undergo oxidation and cyclization to yield unstable endoperoxides which may be transformed either to other short-lived metabolites (thromboxanes, etc.) or to the more stable (but also short-lived) primary prostaglandins. The principal prostaglandins which have been used in immunological studies are those of the E and F series. Their major biosynthetic pathway is



There is a considerable literature on the effect of prostaglandins on immunity which, fortunately, has recently been reviewed in detail (Pelus and Strausser, 1977). The physiological significance of much of this work is difficult to evaluate. Prostaglandins are produced in a wide variety of nonimmunological as well as immunological situations and have many

effects other than those on the immune system. Much of the experimental work describes effects of prostaglandins in tissue culture at concentrations several orders of magnitude larger than the nanogram levels normally found in tissues.

Bearing in mind these limitations, we may summarize the literature as follows.

A. Prostaglandins, Especially Those of the E Series, Inhibit the Immune Response

Inhibition of both T and B cell lymphocyte functions has been described, and different authors (using different experimental systems) have claimed the principal effect of prostaglandins is either on one or the other of the two major sets of lymphocytes.

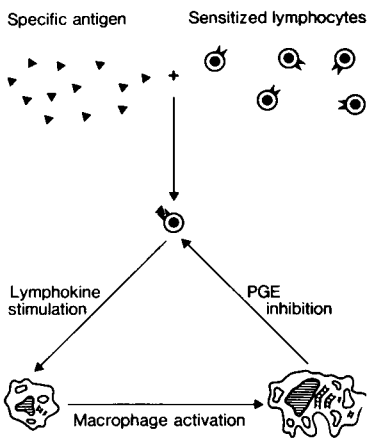
Smith *et al.* (1971b), Offner and Clausen (1974), and Stockman and Mumford (1974) have all reported that E-type prostaglandins (and in some of these papers other prostaglandins) cause a reduction in the ability of phytohaemagglutinin to stimulate T cells. Prostaglandins have been reported also to inhibit *in vitro* T cell cytotoxicity (Lichtenstein *et al.*, 1972; Strom *et al.*, 1974, 1977) and *in vivo* graft rejection (Loose and diLuzio, 1973; Anderson *et al.*, 1975; Strom *et al.*, 1977). Stockman and Mumford found prostaglandin E_2 produced a more than 50% inhibition of phytohaemagglutinin stimulation of [^{3}H]thymidine or [^{3}H]uridine uptake, whereas there was no effect on the response to pokeweed mitogen (which preferentially stimulates B cells).

The opposite conclusion, that E-type prostaglandins inhibit B rather than T cells, was arrived at by two other groups of workers. Quagliata *et al.* (1973), though unable to demonstrate an effect of E-type prostaglandins alone, were able to demonstrate increased immunosuppression (prolonged allograft survival) in mice also treated with procarbazine hydrochloride which is a powerful T cell depressant. They concluded that the principal target of PGE_1 activity was the B cell, both from the preceding observation and a decrease in the numbers of B but not T cells in the spleens of PGE_1 treated animals. An effect on B rather than T cells was also implied by the observation of Zurier and Quagliata (1971) that E-type prostaglandins inhibited the antibody response to sheep red blood cells but not delayed hypersensitivity to PPD. Individual anti-sheep red blood cell antibody-producing lymphocytes (B cells) can be counted by suspending them in a red cell containing agar and measuring the number of lytic plaques formed as a result of antibody-mediated lysis. There are several reports of inhibition of "plaque cell" formation by prostaglandins (Melmon *et al.*, 1974; Plescia *et al.*, 1975a,b).

In the interpretation of all the experiments, possible cytotoxic effects of high ($> 10^{-3}M$) concentrations of prostaglandins *in vitro*, and stress-promoting effects *in vivo* must be carefully considered (Berenbaum *et al.*, 1976).

B. Prostaglandins Can Be Produced By Cells of the Immune System

Ferraris *et al.* (1974) found *in vitro* prostaglandin biosynthesis was increased following antigenic stimulation of spleen cells or unpurified human peripheral leucocytes. After glass-bead purification, known to deplete macrophages, no stimulation of peripheral leucocyte prostaglandin biosynthesis was observed, even though lymphocyte [^{3}H]thymidine uptake was still stimulated. Gordon *et al.* (1976) found that exudate cell populations (60–80% macrophages, 10% lymphocytes) were considerably better than lymph node cell populations ($> 90\%$ lymphocytes) in producing prostaglandin E_2. This suggests the macrophage activated, for example, by lymphokines is a major site of E-type prostaglandin biosynthesis. Since PGEs can also inhibit the activation of lymphocytes and the production of soluble mediators similar to those thought to cause macrophage activation, the possibility exists of a homeostatic mechanism (Morley, 1974):



The existence of such a feedback cycle *in vivo* was supported by the studies of Osheroff *et al.* (1975) and Webb and Osheroff (1976). These workers found that formation of 19 S antibody against sheep red blood cells was enhanced by drugs blocking prostaglandin synthesis. Injection of sheep red blood cells caused, within a few minutes, a 20- to 80-fold,

T lymphocyte-dependent increase in splenic prostaglandin $F_{2\alpha}$. The increase in the prostaglandin $F_{2\alpha}$ content of the thymus (which contains few macrophages) was much smaller (twofold). The rate of increase in prostaglandin $F_{2\alpha}$ following challenge with a soluble antigen (bovine gamma globulin) was slower than following injection of sheep red blood cells, and was bimodal, the later increase occurring at 48 hours. Such timing is closer in order of magnitude to the time required *in vitro* for most macrophage activation processes to be stimulated by lymphocytes (Nath *et al.*, 1973; Meade *et al.*, 1974).

The evidence for this feedback cycle does not preclude the involvement of prostaglandins in other immunological feedback mechanisms. For example, Zimecki and Webb (1976) showed that drugs blocking prostaglandin biosynthesis could also enhance the *in vitro* antibody response to T-dependent antigens. This effect could still be demonstrated in cultures of highly purified B cells implying a regulatory mechanism involving B cells alone. The possibility of prostaglandin-mediated autoregulation of the macrophage must also be included in view of reports that prostaglandins can inhibit the release of lysosomal enzymes from this cell type (Zurier *et al.*, 1971; Ignarro *et al.*, 1973).

Studies on a variety of tissues have suggested that prostaglandin biosynthesis may be regulated by a number of factors including intracellular cyclic AMP levels. However, mitogenic agents capable of stimulating prostaglandin biosynthesis were found by Ferraris *et al.* (1974) not to significantly influence the cyclic AMP content of mouse spleen cells, while cholera toxin and epinephrine, both of which markedly increased the spleen cell cyclic AMP content, did not stimulate prostaglandin release. Another possible factor regulating prostaglandin biosynthesis is the availability for their fatty acid precursors. This is supported by increased prostaglandin biosynthesis by mouse neoplastic cells supplied with exogenous arachidonic acid (Hong *et al.*, 1976).

Activation of macrophages, which have membranes rich in arachidonic acid, leads to increased release of phospholipase A (Munder *et al.*, 1969). This enzyme can cleave phospholipids to release free fatty acids, which will be able to act as precursors for prostaglandin synthetase. Increased levels of extracellular free fatty acids have been demonstrated histochemically in populations of lymph node cells following immunological stimulation (Cyong and Okada, 1976). The cell populations used in these studies were not specifically purified to remove adherent (macrophage-type) cells, but clearly the major cell type was the lymphocyte, and Cyong and Okada (1976) suggested the lymphocyte as the major source of the free fatty acid generating enzyme(s) in their experiments.

Mertin *et al.* (1977) found that removal of the spleen considerably re-

duced the ability of linoleic acid to prolong allograft survival. One interpretation of this result is that injected linoleic acid, which increases arachidonic acid levels in the spleen acts, at least in part, by stimulating prostaglandin biosynthesis of splenic macrophages. Possibly prostaglandins are the major immunoinhibitory agent in these experiments.

VII. Modification of the Immune Response *in Vitro* and *in Vivo* by Exogenous Fatty Acids

In view of the importance of fatty acids as membrane constituents and prostaglandin precursors, discussed in previous sections of this review, it is not surprising that provision of exogenous fatty acids *in vitro* or *in vivo* should modify the immune response. Interpretation of observed effects of fatty acids in terms of what is known of their function at the cellular level is, however, difficult. In determining the particular effect of a fatty acid, not only the nature of that fatty acid but also the form of its administration is critical. Thus, for example, glycerol trioleate given as an emulsion by intravenous injection stimulates reticuloendothelial function, whereas given by mouth it is a reticuloendothelial depressant (Berken and Benacerraf, 1968).

Because fatty acids and their esters are insoluble in water, experiments designed to study the effects of fatty acids *in vitro* or *in vivo* must be carefully designed with respect to the presentation of the fatty acid. For example, fatty acids can be "dissolved" in water by first dissolving them in alcohol, then adding this alcoholic solution to the water. However, it cannot be assumed, even after this procedure, that all the fatty acid is in the aqueous phase without a subsequent analysis. For this reason, some workers have preferred to provide fatty acids *in vitro* bound to albumin, but using this form of presentation for fatty acids complicates analysis of results by adding the rate of dissociation of the albumin–fatty acid complex to those factors which might determine fatty acid activity.

A. Effect of Exogenous Fatty Acids on *in Vitro* Lymphocyte Stimulation

The effect of added fatty acids on lymphocyte stimulation has been studied using dissolved free fatty acids by Mertin and Hughes (1975) and using albumin-bound fatty acids by Weyman *et al.* (1977).

Mertin *et al.* (1974) and Mertin and Hughes (1975) reported that linoleic acid and, to an even greater extent, arachidonic acid suppressed antigen- (PPD) or phytohaemagglutinin-induced increase in lymphocyte

[^{3}H]uridine uptake, while palmitic, stearic, or oleic acid had very little specific activity. Mihas *et al.* (1975), following [^{3}H]thymidine uptake, supported this finding and further showed that inhibitory doses of fatty acids did not affect cell viability. An inhibitory effect of linoleic and arachidonic acids was also reported by Offner and Clausen (1974), who observed an early event following lymphocyte stimulation, the increased incorporation of myo-(2-^{3}H)inositol into phosphatidylinositol. Weyman *et al.* (1975, 1977), while confirming the ability of linoleic and arachidonic acids to inhibit phytohaemagglutinin-stimulated [^{14}C]uridine uptake, did not agree that polyunsaturation was important in determining activity. They also showed appreciable inhibitory activity of the saturated fatty acids, heptadecanoic and stearic acids, as well as the mono-unsaturated oleic acid. In part, this may represent a difference in interpretation of results rather than in the results themselves. Mertin and Hughes (1975) had also reported inhibition of [^{3}H]uridine uptake by stearic, palmitic, and oleic acids, but finding a similar percentage inhibition in unstimulated as well as stimulated lymphocytes had suggested that this inhibition might be nonspecific. Only with linoleic and arachidonic acid was inhibition of phytohaemagglutinin-stimulated uptake significantly different to that of unstimulated cells, and it was for these acids that they postulated an effect on the lymphocyte–antigen or lymphocyte–mitogen interaction. C. Weyman *et al.* were able to repeat this finding (personal communication), but when shorter culture times were employed (42 hours), there was no appreciable depression by any fatty acid of [^{3}H]uridine uptake in unstimulated cells. Effects of saturated fatty acids on uptake in stimulated cells, however, could still be observed (Weyman *et al.*, 1977). It would thus seem that saturated fatty acids can, under appropriate conditions, also inhibit mitogen–lymphocyte interaction. Such an observation does not exclude a specific role for linoleic and arachidonic acid, because nonessential fatty acids can, possibly by acting as competitive substrates, influence the metabolism of essential fatty acids (Lowry and Tinsley, 1966; Dhopeshwarkar and Mead, 1961; Alfin-Slater and Aftergood, 1968). Furthermore, certain saturated fatty acids, particularly lauric and myristic acids, are inhibitors of the conversion of arachidonic acid to prostaglandin E_2 (Robak *et al.*, 1975).

Weyman *et al.* (1977) further reported that when saturated fatty acids were added to an incubation mixture containing unsaturated fatty acids, inhibition by the unsaturated fatty acids was markedly decreased. Myristic acid, which by itself had no effect on [^{3}H]uridine uptake by phytohaemagglutinin-stimulated cells, was particularly effective at abrogating inhibition. Myristic acid also blocked inhibition by saturated fatty acids. This observation made it less likely that the effects of linoleic, arachidonic, and other

fatty acids could be accounted for merely by a toxic action. Investigation of viability by trypan blue exclusion also supported this; Weyman *et al.* could not demonstrate any significant effect on cell viability of the concentrations of fatty acids they employed.

B. *In Vivo* Effects of Fatty Acids

In vivo, the fatty composition of the cells of the immune system can be altered by diet (Tsang *et al.,* 1976; Meade *et al.,* 1978) or by direct injection of fatty acids. Subcutaneous injection is a useful way of providing fatty acids *in vivo* without the toxicity sometimes associated with intraperitoneal administration (Ring *et al.,* 1974), but fatty deposits around the injection site suggest absorption is slow, and analysis is required to give an idea of the changes in serum and organ fatty acid levels which are being produced. Granulomae can also sometimes form at the site of injection, especially when larger quantities of fatty acid are employed. Dietary manipulation of fatty acid levels, while more physiological, has the disadvantage that some of the immunologically most interesting fatty acids are too rare and too expensive to be used as dietary supplements. Intravenous injection of fatty acid or fatty acid ester emulsions has also been used; in this case it appears that the reticuloendothelial system becomes the primary target. The point made earlier, that the same fatty acid can produce different effects depending on its mode of administration, must be emphasized.

The effects of subcutaneous injection of a variety of long-chain fatty acids were studied in mice by Meade and Mertin (1976). We found three types of effect produced following progressively longer periods of fatty acid administration. Initially there was immune activation, then a blocking of reactivity to antigen and finally lymphocytolysis. Most of these studies were made with linoleic acid, although the more expensive arachidonic acid was more effective.

One of the earliest effects of linoleic acid injection was stimulation of [^{125}I]udR uptake in thymus, spleen, and lymph nodes. [^{125}I]udR is an analogue of thymidine, and its uptake is a measure of DNA synthesis, which will be increased with an increased rate of cell division. Other changes associated with immune activation included increased cell division in the bone marrow, and changes in the spleen, such as increased proportion of red pulp and granulocyte precursors, similar to those seen after stimulation by a graft from a different strain of mouse. Autoradiographic analysis showed the lymphocyte was one of the cell types stimulated, although in the spleen other cell types also took up radioactive label following injection of an [^{125}I]udR/[^{3}H]methylthymidine "cocktail."

The idea that linoleic acid could activate the immune system was further supported by comparison between a skin graft from a different strain of mouse, and subcutaneous injection of linoleic acid in their influence on various organ weights. Just as lymph node and spleen weight was increased in skin grafted mice, while thymus weight was decreased, so the same pattern of changes was observed following subcutaneous linoleic acid treatment. The order of activity of fatty acids both in increasing spleen weight (Meade and Mertin, 1976) and decreasing thymus weight (C. J. Meade, unpublished) was arachidonic acid > linoleic acid > α-linolenic acid. Depression of thymus weight (which occurred also in adrenalectomized animals and was therefore not simply a stress effect) was particularly easy to quantitate. Figure 4 shows five fatty acids compared: arachidonic acid, and those fatty acids closer to arachidonic acid along its biosynthetic pathway were most effective. Carbon clearance studies showed arachidonic and linoleic acid, injected subcutaneously, were potent activators of the reticuloendothelial system, while α-linolenic acid had no activity. This contrasts with diLuzio and Blickens' 1966 report of reticuloendothelial system depression by the methyl esters of linoleic acid and arachidonic acid, injected as an intravenous emulsion, and may reflect once again the importance of mode of administration on the biological activity of fatty acids.

Subcutaneous injection of linoleic or (better) arachidonic acid, according to the schedules used in the Meade and Mertin studies, has been shown to raise the level of arachidonic acid in lymphoid tissues (M. Gurr, private communication). The stimulation of lymphocyte division by such injections is therefore compatible with the theories (discussed previously) which

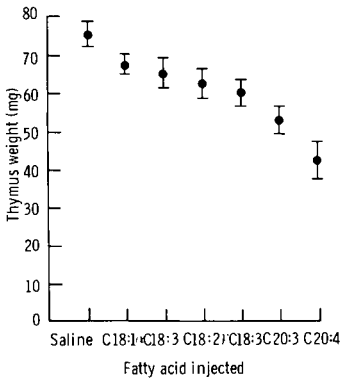


FIG. 4. Effect of different fatty acids on thymus weight. CBA mice received 100 μl of fatty acid spread over 10 days and four subcutaneous injections (C. J. Meade, unpublished).

suggest a role for a rise in membrane arachidonate in lymphocyte triggering. Of course, any antigen will stimulate lymphocyte division, but linoleic and arachidonic acids, being low-molecular-weight normal body constituents, are unlikely to serve as the good antigens, which their powerful *in vivo* activity would imply. Nevertheless, it must be emphasized that many substances not thought to have a role in lymphocyte triggering can also stimulate lymphocyte division *in vivo,* and our results are compatible with, rather than evidence for, the Ferber and Resch theories.

A second group of *in vivo* effects of subcutaneous linoleic or arachidonic acid may be summarized as interference in the interaction of the immune system with antigen. Longer treatment with subcutaneous linoleic acid was shown to reduce the ability of grafts to stimulate lymphocyte division (measured by [^{125}I]udR uptake) in the thymus and lymph nodes (Meade and Mertin, 1976). A parallel may be drawn to the inhibition by this fatty acid of lymphocyte stimulation, *in vitro* (referred to earlier). It has also been shown that certain fatty acids cause a small but significant prolongation of graft survival, the effectiveness again being in the following order: arachidonic > linoleic > α-linolenic acid (Mertin, 1976; Meade and Mertin, 1976). This effect is most readily seen in weak graft rejection systems (e.g., skin from a C3H strain mouse transplanted onto a CBA recipient) but some effect can be observed in strong systems (e.g., A strain skin on a CBA recipient).

It must always be taken into account that a nonspecific stress will also prolong graft survival. In the absence of antioxidants, linoleic and arachidonic acid readily oxidize to toxic peroxides. We were, therefore, careful to aliquot fatty acids used for our studies into small samples kept at $-20°C$. In our experiments, mice were treated with a mean daily dose of 10 μl of fatty acid, and for showing prolongation of graft survival, treatment was continued for 14 or 16 days. Using this regimen, the mortality associated with treatment was negligible and weight gain was similar to, or slightly less than, weight gain of saline-treated controls. By contrast, the grafting procedure itself was always associated with a significant weight loss, presumably resulting from the stress of surgery. It therefore seems that, in our hands, stress associated with treatment was likely to be negligible compared with the stress associated with the surgery of skin grafting.

Another example of inhibition of an immune response by subcutaneous linoleic acid is the reduced accumulation of T cells in the spleens of mice challenged by an intraperitoneal injection of ascitic tumor (Meade and Mertin, 1976). This may represent an abrogation of cell trapping or an effect on stimulation of cell division within the spleen. T cell killing by spleen cells was also reduced (Mertin, 1976).

How are the immunosuppressive effects of linoleic and arachidonic acid to be explained? These effects are preceded by effects associated with immune activation. Levey and Medawar (1966) suggested the concept of *sterile activation* to explain the action of antilymphocyte serum, and one suggestion is that linoleic and arachidonic acid might act similarly to forestall or supplant other immunological commitments. Phytohaemagglutinin, which also nonspecifically activates lymphocytes, also prolongs graft survival (Markley *et al.,* 1967).

Prolongation of allograft survival can also be produced by oral administration of linoleic acid (Ring *et al.,* 1974), and a diet poor in linoleic acid accelerates allograft rejection (Mertin and Hunt, 1976). Detailed studies of the effect of orally administered fatty acids on the lymphoreticular system have not, as yet, been made, and it is not known whether effects on immune organs similar to those seen following subcutaneous administration occur. Preliminary studies (C. J. Meade, unpublished) suggest that depression of thymus weight can also be produced by oral fatty acids, while splenomegaly is not seen.

Prolonged subcutaneous administration of linoleic or arachidonic acids caused damage to the lymphoid organs and particularly the spleen. Time–course experiments suggested that the immunosuppressive effects of these fatty acids could not be solely accounted for by this cytotoxic activity. There was a specificity of damage, severe splenic necrosis being observed without damage to liver tissue (Meade and Mertin, 1976). Free fatty acids are more toxic than the same fatty acids esterified in triglycerides (Morgenstern, 1968; Turnell *et al.,* 1973), and it is tempting to relate the sensitivity of lymphocytes to cytolysis to their high free fatty acid content (Kigoshi and Ito, 1973).

C. Effects of Intravenous Fatty Ester Emulsions

Much of the earliest work on the role of fatty acids in immunity consisted of studies on the effects of injected fatty ester emulsions in experimental animals. Intravenous or intraperitoneal injection of a wide variety of fatty acid or fatty acid ester emulsions caused lymphoid organ necrosis (Shivas and Fraser, 1959). The spleen was a particular target for such emulsions, and this led Stuart (1960) to coin the term *chemical splenectomy* for the effects of intravenous ethyl palmitate emulsions. The dose of emulsion required to produce these effects was relatively large, and the physical form was critical to activity (Shivas and Fraser, 1959). It seems likely, therefore, that emulsified lipids act, in part, in the same way as other colloidal materials which, though chemically inert, can also produce lymphoid tissue necrosis, e.g., colloidal gold (Hahn *et al.,* 1956). The

mechanism of the effect of all these colloids is thought to be via the reticuloendothelial system. Doses of ethyl palmitate able to produce splenic necrosis profoundly depressed reticuloendothelial system function, as measured by colloidal carbon clearance. Since carbon clearance is primarily a function of liver rather than splenic reticuloendothelial function, it is unlikely that changes in this parameter are only secondary to the splenic damage. Further, low doses of ethyl palmitate, too low to cause splenic necrosis, still affect reticuloendothelial function. Changes in macrophages must therefore be considered as possible causes of the changes in the lymphoid tissue.

Although the route and physical form of the injected material is important, the chemical nature of the lipid also appears to play some role. Thus, intravenous injection of 2-oleodistearin, glyceryl tricaprate, or glyceryl oleate produced a mild stimulation of reticuloendothelial function, while intravenous injection of emulsions of a wide variety of other methyl, ethyl, butyl, or cholesteryl esters of long-chain saturated or unsaturated fatty acids depress the reticuloendothelial system (Stuart *et al.*, 1960a,b; Stuart, 1962; Wooles and diLuzio, 1963; Stuart and Cooper, 1963; Stuart and Davidson, 1963; diLuzio and Wooles, 1964; diLuzio and Blickens, 1966; diLuzio, 1972). Therefore it is not enough to view the lipid particles just as an inert food for hungry macrophages which are inactive when they have "overeaten." Rather, the lipid must contribute to the biochemistry of the macrophage and its membrane. This idea is supported by Blickens and diLuzio's 1965 study on the metabolism of methyl palmitate, a potent reticuloendothelial depressant. Twenty-four hours after injection of radioactive methyl palmitate there was little storage of methyl palmitate in liver, lung, or spleen, nor was there a measurable alteration in tissue lipid levels. Therefore, saturation of the reticuloendothelial system by lipid was unlikely. Further, the distribution of radiolabeled methyl palmitate differed from most other colloids known to be cleared by phagocytosis by macrophages, and clearance was not competitively affected by prior injection of colloidal carbon. Hence, it appears phagocytosis is not even a major mechanism for methyl palmitate clearance. Quite how methyl palmitate or other fatty acid esters interact with the macrophage is not known. Structure activity studies made by Cooper (1964) in an attempt to relate stimulation of ^{131}I-labeled triolein clearance *in vitro* and *in vivo* to the chemistry of saturated triglycerides showed no simple relationships, except that *in vivo* activity was related to carbon chain length, deviations from a length of 10 atoms decreasing the ability to stimulate reticuloendothelial function.

Several of these fatty ester emulsions also suppress immune responsiveness. Formation of antibodies following challenge with sheep red blood

cells has been shown to be lowered by intravenous injection of emulsions of methyl palmitate (diLuzio and Wooles, 1964; Wooles and diLuzio, 1963), ethyl palmitate, or cholesterol oleate (Stuart and Davidson, 1964), and a similar result has been obtained using methyl palmitate with a soluble antigen, bovine serum albumin (Ohbuchi, 1968). At the doses employed, these agents also depressed reticuloendothelial system function. The immunosuppressive effects could occur at doses of ester below those at which there was damage to lymphoid tissue and were not necessarily associated with lymphopenia. Morrow and diLuzio (1965) demonstrated an altered clearance of sheep red cell antigen in mice treated with methyl palmitate, so they suggested immunosuppression might be associated with altered processing of antigen by macrophages. Handling of an antigen by macrophages can alter its immunogenicity. The role of macrophages in immune responses is not, as yet, precisely known. Different theories have visualized the macrophage, for example, as an antigen concentration site, particularly for T cell dependent antigens (Lachmann, 1971), or as a site of production of a "super" (possibly RNA associated) antigen which might be transferred to potential antibody-forming cells by intercellular bridges (Cruchaud *et al.*, 1970). Emulsions of fatty esters such as methyl palmitate have been suggested as possible tools to throw light on this problem. For such a purpose it is important to know whether, at the dose level employed in an experiment, the reticuloendothelial system is the only system being affected, for example, by a methyl palmitate emulsion. Kauffman *et al.* (1967), using dogs, found that doses of methyl palmitate below those sufficient to cause reticuloendothelial depression were still able to cause depletion of the follicles of the lymph nodes and spleen. Further, this low dose could inhibit homograft sensitization as measured by second set rejection time of renal transplants following methyl palmitate treatment after first-set grafting. Caution should, perhaps, therefore be exercised before describing any process affected by methyl palmitate as being necessarily macrophage mediated.

VIII. Dietary Fatty Acids, Immunity, and Disease

In previous sections we have described the role of fatty acids in the biochemistry of immune cells and the modification of the immune response by fatty acids. In this section we shall discuss whether any of the work described before may have implications for human disease. Until now, there has been little published work relating to fatty acids, immunity, and disease. We shall therefore concentrate upon (1) identifying diseases in which it might be worthwhile to look for involvement of altered fatty acid

levels in immune responses and (2) presenting alternative, immunological explanations for data relating to dietary fat and disease, usually interpreted without reference to immunology.

Important factors affecting the balance of fatty acids in serum and tissues are diet and the concentrations of hormones such as insulin, adrenalin, etc. Diet is particularly important in determining concentrations of those polyunsaturated fatty acids such as linoleic acid, which cannot be synthesized in the body (Beare and Kates, 1964). Recently, nutrition councils in a number of countries have encouraged the public to increase their consumption of polyunsaturated fats. Recommendations followed work associating diets rich in such fats with decreased incidence of atheroma (Boldingh, 1975). Implications of this recommended change in dietary pattern for long-term immunity to, for example, viruses and tumors remain to be explored. When, in experimental situations, dietary manipulation or injection has been used to alter fatty acid levels, the amounts of fatty acids required to produce immunological effects have been large, and the effects themselves small (Mertin, 1976; Meade and Mertin, 1976; Mertin and Hunt, 1976; Meade *et al.*, 1978). It therefore only seems worthwhile to look for effects of altered fatty acid intake on immunity when there are considerable differences in intake. Patients participating in clinical trials of diets high in polyunsaturated fats for the prevention of cardiac disease may be a group suitable for additional immunological investigation. Another group liable to big differences in polyunsaturated fat intake are babies. Many artificial milks fed to babies are low in polyunsaturated fatty acids in comparison with mother's milk; some manufacturers supplement the milk with linoleate. In early life, when milk is the only source of nutriment, changes in milk composition can alter serum and tissue fatty acid composition. How does this affect the resistance of babies to infection, or, for example, their response to vaccination? Nagai *et al.* (1963) reported higher antimeasles antibody titer, and fewer febrile symptoms, in babies immunized with live measles vaccine and fed linoleate supplemented rather than ordinary artificial milk.

Nonspecific binding of measles virus to a subpopulation of T cells is enhanced by linoleic acid derivative, prostaglandin E_1 (Zurier *et al.*, 1977). If there were an effect of linoleic acid levels on the response to measles virus, then this would be interesting not only because of the possibility of an altered response to vaccination but also because in a chronic disease of the central nervous system, multiple sclerosis, both impaired reactivity to measles virus (Zabriskie, 1975) and lowered serum and lymphocyte linoleate levels have been described (Thompson, 1975; Mahler, 1975). Lowered linoleate serum concentrations are not specific to multiple sclerosis; they occur to some extent in other neurological diseases (Love *et al.*,

1974; Mahler, 1975) and have been described in cystic fibrosis (Rosenlund *et al.*, 1974). Wolfram *et al.* (1974) described a fall in serum cholesteryl linoleate in patients after major surgery. Sudo (1962) described an essential fatty acid deficiency in rats with an experimental streptococcal infection. Possibly lowered serum concentrations of essential fatty acid represent a response to prolonged stress.

In the following sections we shall discuss (in a deliberately one-sided way) whether any of the data accumulated on fatty acids and heart disease, tumor growth, or multiple sclerosis might be interpretable in an immunological context.

A. Cardiovascular Disease

The increased levels of antimilk antibodies in the serum of patients recovering from myocardial infarction (Davies *et al.*, 1974) has led to suggestions of an involvement of autoimmune processes in atheroma. However, as yet there is no evidence that such increased antimilk titers are not an effect rather than a cause of cardiac disease (e.g., a result of increased permeability of the intestine to denatured milk antigens). It seems premature to involve such postulated autoimmune processes in explanations for protective effects of polyunsaturated acids. In patients with cardiovascular disease associated with increased fatty acid serum concentrations, Dil'man (1976) has found decreased *in vitro* lymphocyte responsiveness to phytohaemagglutinin; normalization of the serum fatty acid concentrations after treatment with phenformin resulted in restitution of the immune response.

B. Tumor Growth

Influences on the immune system provide one explanation for the known effects of alterations in the dietary fatty acid content on the incidence of tumors in experimental animals following treatment with carcinogens. Carroll and Khor (1975) found that high-fat diets predisposed rats to mammary tumors after a single oral dose of 7,12-dimethylbenz-2-anthracene (DMBA). Unsaturated fats were more effective than saturated fats, a finding confirmed by Hopkins *et al.* (1976). The latter workers found that a diet rich in polyunsaturated fats was effective even when begun after DMBA administration but ineffective when fed before administration of the carcinogen with a switch to a saturated fat-rich diet afterwards. Carroll and Khor (1975) could obtain an enhanced yield of mammary tumors following DMBA administration when the switch to a corn oil-supplemented (i.e., polyunsaturated-fat-rich) diet was made as much

as two weeks after administration of carcinogen. Rao and Abraham (1976) found enhanced growth of a mammary adenocarcinoma transplanted into mice fed a linoleate-rich diet. It is therefore likely that the effect of polyunsaturated fat-rich diets is on the survival and proliferation of tumor cells (in which immune mechanisms may be involved) rather than on the initial event of neoplastic transformation. The opposite effect, increased tumor resistance in mice fed an essential fatty acid-deficient diet, has been shown following methylcholanthrene treatment of mice by Mertin and Hunt (1976). However, the effects of high- or low-fat diets on tumor growth may merely reflect a more general effect of availability of nutrients, either to the tumor itself or to cells of the immune system. Other changes in nutritional status, such as protein–calorie malnutrition, have also been shown to alter the rate of growth of tumors, and immunological factors have been implicated in these effects (José and Good, 1973).

Chemically induced tumors are extremely antigenic in comparison with those which arise spontaneously, and their growth can be affected by agents which alter the immune response. There is doubt about the importance of immune mechanisms in the control of spontaneous tumor growth, despite the attractiveness of concepts of *immune surveillance* (Burnet, 1970). Except for the special cases of lymphoreticular or viral induced tumors, there is not a greatly increased tumor incidence either in patients immunosuppressed after transplantation, or in immunodeficient mice. Therefore, studies on the effect of diet on chemically induced carcinogenesis are not necessarily applicable to the different situation of "spontaneous" tumeriogenesis. Conclusive proof that dietary fats alter either morbidity or mortality from tumors in man has yet to be obtained. Epidemiological studies are complicated by associations between fat consumption and other factors such as obesity. Pearce and Dayton (1971) reported a slightly higher incidence of neoplasms in men eating a diet high in polyunsaturated fats as part of a trial for the prevention of heart disease. This result was on the borderline of significance ($p = 0.06$). No such increase has been observed in other, larger trials (Ederer *et al.*, 1971; Miettinen *et al.*, 1972). The Pearce and Dayton trial included elderly men who, if protected from atherosclerosis, were likely to die of some other cause, and cancer is the next most common cause of death in such a population.

C. Multiple Sclerosis

Swank, on the basis of epidemiological data suggesting involvement of dietary factors in multiple sclerosis (Swank *et al.*, 1952), treated multiple sclerosis patients with a diet low in fat and high in oils (i.e., low in saturated but rich in polyunsaturated fats). He claimed his diet had, over

20 years' use, had a beneficial effect on the course of the disease (Swank, 1970). However, Swank's study made no comparison with a proper control group, only with relapse and survival rates reported in the literature. A better trial was the controlled double-blind trial reported by Millar *et al.* (1973). The diet of multiple sclerosis patients was supplemented with either linoleate-rich sunflower seed oil, or with a smaller quantity of oleate-rich olive oil. Relapses tended to be less severe and of shorter duration in the linoleate-supplemented group than in those receiving the oleate mixture, but clear evidence that treatment affected the overall rate of clinical deterioration was not obtained. Even this trial has been the subject of criticism (Vessey, 1975), and more double-blind trials are required to establish conclusively whether polyunsaturated fatty acids are beneficial in multiple sclerosis. There are many suggestions for the mechanism of the postulated beneficial effects of polyunsaturated fatty acids in this disease, and our recent review (Mertin and Meade, 1977) discusses these in detail. One possible mechanism is via the immune system. There is some evidence for involvement of autoimmunity in multiple sclerosis:

1. The presence of lymphocytes and other mononuclear cells in the early perivascular "cuffings" and at the edge of plaques in the central nervous system (Adams, 1977).
2. Similarities between the lesions in multiple sclerosis and iatrogenic allergic encephalomyelitis following injection of rabies vaccine contaminated with nervous tissue (Uchimara and Shiraki, 1957).
3. Presence of oligoclonal IgG in the cerebrospinal fluid (Link, 1973).
4. Increased frequency of multiple sclerosis in subjects having certain histocompatibility antigens on the surface of their lymphocytes, a feature of several autoimmune diseases (Batchelor, 1977).

There is no ideal animal model for multiple sclerosis. Injecting susceptible animals, together with an adjuvant, with homogenates of central nervous system tissue, or a protein extracted from such tissue, termed *myelin basic protein,* induces an allergic encephalomyelitis. This experimental autoimmune disease resembles multiple sclerosis in part clinically, and the observed demyelination in the central nervous system is here also associated with perivascular infiltration of mononuclear cells. Clausen and Møller (1969) showed a diet deficient in essential fatty acids potentiated the ability of injected brain homogenates to produce experimental allergic encephalomyelitis in rats. Selivonchick and Johnston (1975) confirming this also showed a protective effect of oral ethyl linoleate in rats fed a basic diet which was fat deficient. Meade *et al.* (1978) were able to show a protective effect on linoleic acid in guinea pigs fed a standard diet that

was adequate in essential fatty acids, but which was sufficiently low for it to be possible to significantly raise serum linoleate levels by feeding.

These experiments can be interpreted in many ways. Clausen and Møller considered their observations largely in terms of altered myelin fatty acid composition affecting myelin stability. Unfortunately, the fatty acid analyses they use to support their hypothesis disagree with other reported figures for brain lipid composition. Selivonchick and Johnston (1975) found the lipid composition of myelin was little changed by their dietary treatment; Meade *et al.* (1978) reported their feeding schedule produced little change in brain fatty acid composition in normal animals, although serum and lymph node composition were altered. It therefore seems difficult to explain the effect of dietary fats on experimental allergic encephalomyelitis in terms of gross changes in myelin composition, although changes in particular membranes forming a small proporton of the total lipid (e.g., those forming the blood brain barrier) cannot be excluded. One alternative

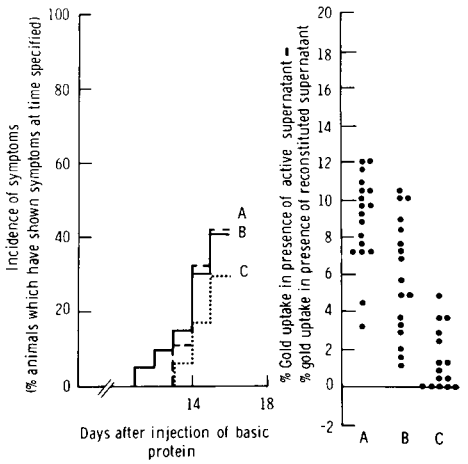


FIG. 5. Effect of linoleic acid on the clinical signs of experimental allergic encephalomyelitis, and the ability of isolated lymph node cells to produce macrophage-activating lymphokines on incubation with basic protein. Each spot represents one animal. Macrophage activation is assayed by the capacity of a macrophage monolayer to take up radioactive colloidal gold. The "active" supernatant is prepared by incubating lymphocytes with basic protein. The reconstituted supernatant is a control. Lymphocytes are incubated without basic protein, which is added only at the end of the culture. Group A = guinea pigs fed linoleic acid from 7 days before until 7 days after injection of basic protein in complete Freund's adjuvant. Group B = guinea pigs injected with basic protein in adjuvant but not fed a dietary supplement. Group C = guinea pigs fed linoleic acid from day 7 until they were killed on day 15. Comparison of groups B and C; significant difference in gold uptake $0.001 > p$. From Meade *et al.* (1978).

hypothesis sees the protective effect of linoleate resulting from an effect on the immune system. In support of this, Meade *et al.* (1978) found that feeding guinea pigs linoleic acid according to a schedule which reduced clinical and histological signs of experimental allergic encephalomyelitis also reduced the ability of isolated lymph node cells to respond to basic protein *in vitro* by production of macrophage-activating substances (Fig. 5).

IX. Summary

Since diLuzio's 1972 review on lipids and immunity in this series, new perspectives have opened. In biochemistry, the importance of fatty acids as membrane components and as prostaglandin precursors is becoming increasingly recognized. Ferber and Resch (1976) have provided a coherent and testable model for lymphocyte activation based on alterations in membrane fatty acid composition that has to be submitted to further investigations applying also, for example, inhibitors of lymphocyte activation. Lipids as lymphocyte membrane components have, up to now, been neglected by immunologists in favor of the protein components. One reason for this is the difficulty in preparing specific antisera against lipids. This contrasts to the relative ease with which immunological methods can be applied to the investigation of membrane proteins, for it was, above all, the use of fluorescent and cytotoxic antisera that allowed their detailed investigation. By contrast, the techniques of lipid analysis are rarely available in immunology laboratories.

We have described the effects of fatty acids on immune cells *in vitro* and *in vivo,* but it is clear we are still largely at the stage of describing phenomena rather than understanding them. We may summarize by saying that a wide variety of fatty acids produce effects on both the lymphoid and reticuloendothelial systems, the actual effects produced depending on the method of administration as well as the chemical nature of the fatty acid. Only when examining the effects of subcutaneously injected unsaturated fatty acids is a pattern discernible, increasing dosages of polyunsaturated fatty acids producing successively immune activation, immune inhibition, and lymphocytolysis, and arachidonic acid and its precursors being more active than other fatty acids tested. Arachidonic acid is also an immediate prostaglandin precursor, and plays a central role, according to the hypothesis of Ferber and Resch (1976), in lymphocyte activation.

With regard to fatty acids, diet, and disease, we consider it too early yet to say whether a role for fatty acids in the biochemistry of immune cells, or effects of fatty acids *in vivo,* have any relevance to human disease. The reticuloendothelial-suppressing and chemical splenectomizing actions

of fatty acid ester emulsions have led to suggestions for their therapeutic use in acquired hemolytic anemia (Stuart and Davidson, 1963). The effect of fatty acid esters on reticuloendothelial function may be relevant if these esters are employed to form part of the liposomes used to entrap therapeutic drugs (Gregoriadis, 1977).

Prostaglandins are at present under investigation as suppressors of transplant rejection (Strom *et al.*, 1977). In other fields, natural prostaglandins (as well as thromboxanes and other intermediates) have, until now, frequently proven too unstable and possessing too wide a spectrum of actions to be useful drugs themselves. Research is moving toward the use of more stable synthetic prostaglandins, and as an alternative approach, to prostaglandin precursors (i.e., essential fatty acids) and the relevant biosynthetic mechanisms. The immunosuppressive action of polyunsaturated fatty acids alone is probably too limited to make them useful drugs in, for example, the treatment of transplant rejection, although they may be useful as additional—and nontoxic—constituents in a conventional immunosuppressive therapy (McHugh *et al.*, 1977). They also may find application where their ability to pass through membranes impermeable to water soluble drugs is of advantage. Such barriers include the skin (Press *et al.*, 1974; Friedman *et al.*, 1976) and the blood brain barrier.

To conclude, we hope that we have shown in this review that immunology may have a place in fatty acid research. We only hope that, in making this point, we do not blind the reader to the reality that effects on immunity represent only one of many ways in which fatty acids might be relevant to disease.

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