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Modulation of Inflammation and Immunity by Cyclic AMP

Receptors for vasoactive hormones and mediators of inflammation regulate many leukocyte functions.

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Fifteen years ago Sutherland and Rall identified adenosine 3′,5′-monophosphate (cyclic AMP) as an intracellular "second messenger" mediating epinephrine's stimulation of hepatic glycogenolysis (*1*). The discovery of cyclic AMP triggered a geometrically increasing series of investigations (*2*) which rapidly encompassed much of endocrinology and spread to such diverse fields as neurophysiology, microbiology, plant biochemistry, and the study of cell growth and differentiation (*3*). More recently, cyclic AMP research has begun to intersect with the equally rapidly growing field of immunology. In the past 3 years cyclic AMP, acting as a second messenger for vasoactive hormones and mediators of inflammation, has been shown to modulate a wide variety of immune processes in vitro and in experimental models of immediate and delayed hypersensitivity and the humoral antibody response. Both the newly deciphered immunologic "messages" transmitted by cyclic AMP and the hormones that appear to initiate them form a distinctive and consistent pattern: In each in vitro system, cyclic AMP inhibits an immunologic or inflammatory action of one or another subclass of leukocytes; in each system, production of cyclic AMP is stimulated by β-adrenergic catecholamines, histamine, and the E-series prostaglandins.

In this article, we propose to summarize the experimental evidence that cyclic AMP may regulate leukocyte functions, and to outline a resulting hypothesis: *Certain hormones and mediators of inflammation act in vivo to regulate the character and intensity of inflammatory and immune responses; this regulation is mediated by a general inhibitory action of cyclic AMP on immunologic and inflammatory functions of leukocytes.* Three merits of this hypothesis should become evident. (i) The hypothesis incorporates a large body of experimental evidence from many laboratories. (ii) Its predictions can be tested by available immunologic techniques. (iii) It places the consistent "inhibitory" effects of cyclic AMP in leukocytes squarely in the mainstream of current understanding of the cyclic AMP system.

The messenger function of cyclic AMP was initially thought of primarily as a "turn-on" switch whereby hormones could stimulate the liver, heart, adipose tissue, or target endocrine glands such as the thyroid and adrenal (*3*). Now we know, however, that cyclic AMP can inhibit or "turn off" several cell functions, such as the aggregation of platelets (*4*) or the growth of normal or malignant cells in culture (*5*). Thus the diverse messages transmitted by cyclic AMP may be "on" or "off" in a particular cell type, but seem to constitute crucial links in homeostatic regulation of the milieu interieur. Immunologic responses to antigens may be indispensable protective mechanisms but may also threaten the survival of the host if they are not controlled, as in human diseases such as systemic lupus erythematosus. Our hypothesis suggests that certain vasoactive hormones, mediators of inflammation, and cyclic AMP serve to protect the host from the dangerous consequences of an unregulated immune response.

Leukocyte Effector Functions

In 1968 Lichtenstein and Margolis presented in *Science* (*6*) the first evidence implicating cyclic AMP in control of leukocyte function: Epinephrine and theophylline, agents that, respectively, stimulate synthesis or decrease degradation of cyclic AMP in many tissues, prevented the antigen-induced release of histamine from leukocytes of patients with ragweed hay fever. In this in vitro model of the immediate hypersensitivity reaction, histamine secretion (*7*) is triggered by the combination of antigen with specific immunoglobulin E (IgE) antibody bound

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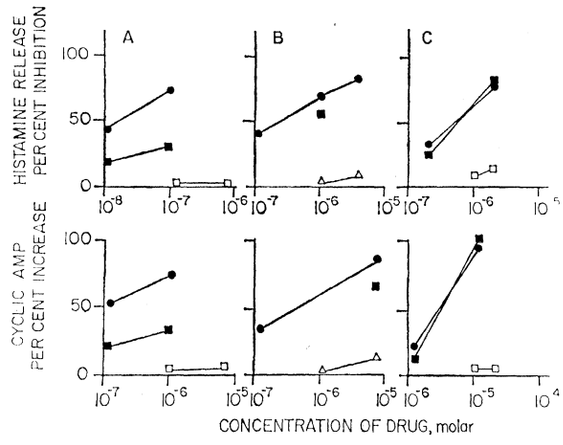

Fig. 1. This figure represents a summary of a number of experiments (*6, 8, 9*) which show the parallel effects of the catecholamines, prostaglandins, and histamine, together with appropriate antagonists, on the inhibition of histamine release and the increase in cyclic AMP in human leukocyte populations. (A) The effects of isoproterenol (●) (largely β-adrenergic), norepinephrine (■) (largely α-adrenergic), and isoproterenol together with propranolol (□), a β-adrenergic antagonist. Propranolol at these concentrations has no effect by itself. (B) The effects of prostaglandins E_1 (●) and $F_{2\alpha}$ (△). Propranolol demonstrates no antagonism to prostaglandin E_1 (■). (C) The effect of histamine (●), or histamine together with an H1 antagonist (diphenhydramine) (■), or an H2 antagonist (burimamide) (□) (*9*). Only the H2 antagonist blocks the effects of histamine in this system.

to membranes of basophilic leukocytes. Subsequent evidence that cyclic AMP is the intracellular inhibitor of histamine release (Table 1) may be summarized briefly as follows:

1) Three classes of endogenous hormones—β-adrenergic catecholamines, E prostaglandins, and histamine itself—stimulate accumulation of cyclic AMP in leukocytes by activating adenylate cyclase, the enzyme which converts adenosine triphosphate to cyclic AMP; the same agents, at similar concentrations, prevent antigen-induced release of leukocyte histamine (*8*) (Fig. 1). Appropriate pharmacologic antagonists, such as propranolol (a β-adrenergic blocking agent) and burimamide, a newly developed antihistamine, prevent both the rise in cyclic AMP and the inhibition of histamine release caused by their respective agonists (*8, 9*). Congeners of the hormones, such as α-adrenergic amines (for example, phenylephrine) or the F prostaglandins, had little or no effect on either histamine release or leukocyte cyclic AMP (*8*).

2) Methylxanthines, such as theophylline, inhibit leukocyte phosphodiesterase, the enzyme that degrades cyclic AMP, and potentiate the effects of hormones on both cyclic AMP accumulation and inhibition of histamine release (*8, 10*).

3) Dibutyryl cyclic AMP, an analog of the endogenous nucleotide, inhibits histamine release.

4) Cholera enterotoxin, a nonspecific activator of adenylate cyclase in gut mucosa and many mammalian cells (*11*), causes a delayed increase in leukocyte cyclic AMP whose time course correlates perfectly with toxin-induced inhibition of histamine release. Both effects are inhibited by specific toxin antagonists (*12*).

Assem and Schild, Austen, Ishizaka, and their co-workers have extended these findings to the antigen-induced release of histamine and slow reacting substance of anaphylaxis (SRS-A) from passively sensitized human and monkey lung (*13*). The same pharmacologic agents are active in these lung preparations, where they presumably inhibit release of inflammatory mediators from tissue mast cells. As in leukocytes, the drug-induced increase in lung cyclic AMP correlated with inhibition of histamine and SRS-A release (Table 1).

Antigen-induced release of histamine or SRS-A may be thought of as an effector mechanism by which an immunologic response (synthesis of specific IgE antibody) is directly linked to inflammation. During the past 2 years, cyclic AMP has been shown to control at least four additional immunologic or inflammatory "effector functions" of other leukocyte subtypes, including neutrophils and both thymus-derived (T) and bone marrow–derived (B) lymphocytes. These findings, summarized in Table 1, are described briefly below.

Cell-mediated immune responses, typified by delayed hypersensitivity skin reactions and by homograft rejection, are thought to reflect activities of T (thymus-dependent) lymphocytes (*14*). By means of an in vitro model of allograft rejection, T cells from the spleens of mice immunized with allogeneic cells have been shown to effect cytolysis of cells bearing the appropriate alloantigen (*15*). In this system, Henney and his colleagues found that all the pharmacologic agents that inhibited histamine release also suppressed T cell–mediated cytolysis and caused, in a parallel manner, increases in the cyclic AMP content of the lymphocyte effector cell population (*12, 16*). These findings have been confirmed with lymphocytes from the

Table 1. Hormonal control of effector cell function. The plus sign (+) indicates that all pharmacological agents inhibited the leukocyte function or caused increased accumulation of cyclic AMP in the cells. The minus sign (−) indicates that the agent did not cause inhibition. Abbreviations: PG's, prostaglandins; cAMP, cyclic AMP; and SRBC, sheep red blood cells.

Species	Reaction	Inhibitory effects of:				Correlation with cAMP	Reference
		Histamine	β-Catecholamines	PG's	Cholera toxin		
		Immediate hypersensitivity					
Human basophil	Histamine release	+	+	+	+	+	(*6, 8, 9, 12*)
Human and monkey lung (mast cell)	Histamine, SRS-A release	?	+	+	?	+	(*13*)
		Acute inflammation					
Human neutrophil	Release of lysosomal hydrolases	+	+	+	—	?	(*24, 25*)
		Cell-mediated immunity					
T lymphocyte of: Mouse and rat	Immune cytolysis	+	+	+	+	+	(*12, 16, 28*)
Man	Interferon production	?	+	+	+	+	(*19*)
		Humoral immunity					
B lymphocyte/plasma cell (mouse)	Plaque formation on SRBC	+	+	+	+	+	(*23*)

rat (*17*). In this experimental model the cyclic AMP–active drugs prevent cytolysis by affecting the function of the lymphocyte, rather than by protecting the target cell (*12, 16, 17*).

Although it is not known whether target cell lysis is caused by secretion of a soluble mediator from the lymphocyte, "sensitized" T cells do appear to synthesize a plethora of other substances when exposed to specific antigen (*18*). Production of one such substance, interferon (a protein that blocks viral replication in mammalian cells), is prevented by exposure of activated lymphocytes to cholera enterotoxin and other cyclic AMP–active substances (*19*). The best characterized of these lymphocyte products, migration inhibitory factor (MIF) (*20*), is thought to be responsible for the characteristic collection of mononuclear cells in delayed hypersensitivity skin reactions. Although the possible effect of cyclic AMP on secretion of MIF has not been studied, mixtures of theophylline and isoproterenol did prevent the effect of MIF on macrophage migration (*21*).

The other major lymphocyte subclass, termed B lymphocytes, proliferate as a result of antigenic stimulation and eventually differentiate into antibody-secreting plasma cells. Using Jerne's hemolytic plaque assay (*22*) for the in vitro detection of antibody-forming cells, Melmon and his colleagues (*23*) have found that the number of demonstrable antibody-forming cells in splenic lymphocyte populations of immunized mice decreases in the presence of β-adrenergic catecholamines, histamine, and prostaglandins, as well as methylxanthines, dibutyryl cyclic AMP, and cholera enterotoxin. These drugs appeared to inhibit either the production or the secretion of antibody. Lymphocyte cyclic AMP increased appropriately in response to the same agents.

The polymorphonuclear neutrophil, which plays a central role in acute inflammatory responses, is in some ways an immunologic "effector cell" as well, by virtue of its participation in reactions triggered by immune complexes (antigen-antibody) and the complement system. When neutrophils phagocytize particles or immune complexes, they release a portion of their lysosomal contents into the extracellular medium, an event that may be responsible for certain features of acute inflammation (*24*). Weissman and his co-workers have reported that prostaglandin E_1 (PGE_1), methylxanthines, and cyclic AMP inhibit phagocytosis-induced release of β-glucuronidase and other lysosomal hydrolases by human neutrophils in vitro (*24*). More recently, by preventing phagocytosis with cytochalasin B, these workers have been able to trigger secretion of neutrophil hydrolases with immune complexes: prostaglandins E and A (but not F), histamine, and β-adrenergic catecholamines all inhibited hydrolase secretion (*25*). Although it is not certain that the hormonal inhibition in this system was mediated by neutrophil cyclic AMP (*26*), the pattern of pharmacologic inhibition of neutrophil function is strikingly similar to those described with histamine release and T and B lymphocyte function.

From these experimental models (Table 1), two points emerge, both pertinent to our general hypothesis relating cyclic AMP to regulation of immune and inflammatory responses in vivo: (i) Receptors for a strikingly consistent array of vasodilating hormones—histamine, β-adrenergic catecholamines, and the E series prostaglandins—have been demonstrated in four subclasses of leukocytes. (ii) In contrast to cyclic AMP's stimulation of secretion in other cells [for example, salivary gland and pancreatic islet cells (*3, 27*)], the exogenous or endogenous nucleotide consistently inhibits secretory events in leukocytes thought to be necessary for expression of immune responses. These two findings have stimulated investigation in complementary directions. Several laboratories are beginning to explore the immunologic implications of receptors for vasoactive hormones in leukocytes, while others are investigating the effects of cyclic AMP on early states of the immune response, prior to its expression via antibody production; release of cytotoxic substances, MIF, and other products of activated lymphocytes; or secretion of inflammatory mediators.

Lymphocyte Hormone Receptors

By pharmacological criteria (the use of congeners and specific pharmacological antagonists), the experiments outlined above have demonstrated the presence of specific and distinct receptors for prostaglandins, catecholamines, and histamines on lymphocytes. A few years ago this surprising conclusion could hardly have been predicted, and its physiological significance is obviously open to question. Why should lymphocytes be able to respond to a group of vasoactive hormones usually thought of as physiologic regulators of the contraction of smooth muscle? Although the question has not been answered, several experimental approaches have provided a useful hint. It is beginning to appear that receptors for vasoactive hormones are not present in random fashion on all lymphocytes, but may in fact develop concomitantly with the commitment of a clone of immunocompetent cells to expression of either cell-mediated or humoral immune responses.

The possibility that lymphocytes mediating cellular immunity develop receptors for histamine after immunization was suggested by an almost chance observation of Plaut *et al.* (*28*). They initially observed an unexplained day-to-day variation in the ability of histamine to inhibit the specific cytolysis of target cells by "sensitized" mouse splenic lymphocytes. Reexamination of their data revealed that the spleen cells had been tested for cytolytic activity at varying times (8 to 17 days) after immunization. When this variable was controlled, they found a reproducible increase in the inhibitory effect of histamine with increasing time after immunization. Ten days after immunization histamine ($10^{-5}M$) caused only approximately 10 percent inhibition of cytolysis, a value which gradually rose to more than 50 percent by day 16 (Fig. 2). In contrast, dibutyryl cyclic AMP, cholera enterotoxin, and PGE_1 caused consistent inhibition of cytolytic activity, regardless of the time elapsed since immunization. Such experiments suggest that the cytolytic mechanism itself is inhibited by cyclic AMP at any time, but that histamine receptors gradually develop on "killer lymphocytes" only after immunization (*28*).

Parallel evidence for development of hormone receptors—on lymphocytes responsible for humoral immunity (B cells)—was obtained by the quite different approach of Melmon and his co-workers (*29, 30*). They conjugated vasoactive hormones covalently to a carrier peptide or protein (rabbit or bovine serum albumin), which was then coupled to Sepharose beads; the insolubilized hormone conjugates were found to bind most human blood leukocytes and a proportion of mouse splenic leukocytes, but did not stimulate accumulation of cyclic AMP in the

Table 2. Direct plaque-forming cell responses in irradiated (BALB/c × C57BL/6)F_1 mice injected with mixtures of syngeneic spleen cells separated on various types of Sepharose columns. RSA, rabbit serum albumin; PFC, plaque-forming cells.

Type of Sepharose column*	Spleen cells transferred (No.)		Direct PFC per recipient spleen (mean ± S.E.)
	Nonadherent	Eluted	
None	5×10^6	0	1210 ± 149
RSA	5×10^6	0	1190 ± 132
Histamine-RSA	5×10^6	0	4050 ± 547
Histamine-RSA	0	1×10^6	410 ± 153
Histamine-RSA	5×10^6	1×10^6	1290 ± 407

* Sepharose preparations and column chromatography are described in the text and in (*29–32*).

cells. The experimental implications of this approach are twofold: (i) It represents an attempt at detection of hormone receptors on cell membranes by criteria independent of measuring hormonal effects on cyclic AMP or on cell function. (ii) The binding of certain cells to insolubilized hormone may allow the separation of cells that have membrane receptors for endogenous amines from cells that do not have such receptors; then the immunologic functions of the two cell populations can be compared.

The specificity of leukocyte binding by insolubilized hormone conjugates is being explored: It was demonstrated that the hormone (histamine or catecholamine) was critical for binding of cells, since beads alone or beads linked to carrier proteins or peptides did not bind cells. Binding of cells to histamine-carrier-Sepharose or norepinephrine-carrier-Sepharose was blocked or reversed by appropriate hormone antagonists (antihistamines or β-adrenergic blocking agents, respectively), or by the corresponding free hormone itself (*30*). Such experiments are compatible with the idea that the binding phenomena observed with the insolubilized hormones were dependent upon at least a portion of the cell membrane receptors for the amines. We know that stimulation of mixed leukocyte populations by the free amines results in increased cyclic AMP synthesis. Therefore, if the binding to conjugates were by natural receptors for the corresponding free amine, then the cells which do not bind to hormone-bead preparations should not have such receptors and should not synthesize cyclic AMP in response to the stimulation with the free (soluble) hormone. Under certain circumstances, when mouse splenic leukocytes were used, this proved to be the case (*31*).

The second implication, that cells capable of binding to hormone-carrier-Sepharose would be functionally different from those that did not, was investigated in the Jerne hemolytic plaque system for measuring production of antibody to sheep red blood cells (SRBC). Splenic leukocytes from two mouse strains previously immunized with SRBC were mixed with beads bearing insolubilized hormone. The mixture was poured into chromatographic columns, and bound cells were separated from free cells. A large proportion (up to two-thirds) of the antibody-producing cells were retained by columns of insolubilized histamine, isoproterenol, epinephrine, or PGE_2. Antibody-forming cells were not retained by columns of insolubilized norepinephrine and $PGF_{2\alpha}$, or by Sepharose alone and Sepharose-carrier alone (*31*). The ability of insolubilized hormones to bind antibody-forming cells correlated very well with the ability of the same soluble hormones to inhibit hemolytic plaque formation by the cells. For example, both effects were observed with PGE's but not PGF's, and with β- but not α-adrenergic catecholamines (isoproterenol and epinephrine as compared to norepinephrine and phenylephrine) [see above and (*23*)].

These "column" experiments suggest that cells actively producing antibody possess surface receptors for hormones. Other experiments, performed by Shearer and co-workers, using the same insolubilized hormone preparations, suggest that precursors of antibody-forming cells do not have such receptors (*32*). Spleen cells from unimmunized mice were injected into lethally irradiated, syngeneic recipients. When the recipient animals were immunized with SRBC, a portion of the transferred cells proliferated and differentiated into antibody-forming cells, which were detected by the hemolytic, plaque-forming cell assay (*33*). If the precursors of antibody-forming cells in the donor spleens expressed membrane receptors for insolubilized hormones, filtration of the cells over insolubilized-hormone columns should have reduced the immune response potential of the unbound cells because of retention of precursor immunocytes. This did not occur (Table 2), suggesting that the transferred precursor cells, unlike cells actively forming antibody, did not express amine receptors (*32*). The implication is that activation of antigen-specific mouse lymphocytes by immunization with SRBC leads to expression of amine receptors on antibody-producing progeny of the activated cells (*34*).

Shearer's transfer experiments produced one surprising result: If the column procedure with hormone conjugates had only failed to bind precursors of antibody-forming cells, then no change should have been detected in plaque-forming cell responses in the recipients; instead, the plaque-forming cell responses were consistently increased if donor cells had been passed over a histamine-Sepharose column, as compared with either cells that were not passed through a column ("no column") or cells which passed through protein-Sepharose columns containing no hormone (Table 2) (*32*). The most likely explanation of this phenomenon is that the insolubilized histamine selectively attracted and removed a subpopulation of cells that could suppress proliferation of antibody-forming cells in the recipients (*35*). If such a regulator-suppressor cell were removed by the histamine-conjugate columns, then the addition of cells eluted from the column to those that were not retained should result in an antibody-forming cell response in the recipients not different from that of the controls. The preliminary observations shown in Table 2 (compare line 3 with line 5) suggest that the elevated response generated in vivo by the cells which did not adhere to hormone-carrier columns was due to removal of a regulator cell (or substance, or both), rather than to a selective concentration of immunocompetent precursor cells. The precise identity of the "regulator" or "suppressor" cells and the mechanism of their suppressive action remain to be elucidated. At this point, it is not known whether the suppression is confined only to responses to SRBC antigens, or whether it may be a more general phenomenon. In any event, the phenomenon observed provides additional evidence that hormone receptors, as detected by binding to insolubilized hormones, are not randomly distributed

among lymphoid cells, but probably are present on discrete subpopulations of cells with distinct immunological functions. The finding thus sets the stage for possible hormonal control of immune responses, related to cell-cell interactions.

Early Stages of the Immune Response

Early events in the production of an immune response to antigen include initial antigen processing (which appears to involve macrophages in some circumstances) and antigen-lymphocyte interaction (the specificity of which involves a membrane-associated antigen-recognition unit). As a result of the interaction with antigen, the lymphoid system is either rendered "tolerant" (by a mechanism as yet unknown), or lymphoid cells are induced to differentiate into antibody-forming plasma cells or "effector" T cells (or both). Most antigens stimulate both humoral and cell-mediated immune responses concomitantly, but some antigens have been found to initiate antibody formation with no demonstrable cell-mediated immunity (for example, pneumococcal polysaccharide III), and others (for example, dinitrochlorobenzene when administered as a skin "paint") potentiate cell-mediated immune responses in the apparent absence of antibody formation. The reasons for these phenomena are unclear, but may relate to factors associated with antigen-lymphocyte interaction. Such early events in the immune response appear to demand communication between different cell types. Thus, interactions between B and T cells, which have been shown to be important in amplification of antibody formation to many kinds of antigens (*36*), can apparently also lead, in some circumstances, to suppression of the immune response (*37*). Other recent evidence suggests that interactions between subpopulations of T cells can lead to a synergistic amplification of the immune response (*38*), or in other circumstances can cause immune suppression (*39*). The possibility that cyclic AMP plays a role in such cell-cell communication is obvious, but has not yet carefully considered.

One study has made an attempt to examine the effect of cyclic AMP at the earliest stage of the humoral immune response, the first contact of lymphocytes with antigen (*40*). Unsensitized mouse spleen cells were exposed in vitro to the multichain synthetic polypeptide antigen, (Tyr, Glu)-Pro-Lys, and subsequently were injected (after being washed) into irradiated syngeneic recipient animals. Contact of the cells with antigen before transfer specifically suppressed the serum antibody response to subsequent stimulation with the same antigen in the recipient animals. If the lymphocytes were exposed to PGE_1 in vitro, during the first contact with antigen, suppression of the later response to antigen was blocked (*40*). Later experiments showed that histamine, dibutyryl cyclic AMP, cholera enterotoxin, and methylxanthines also blocked the antigen-induced immune suppression, in parallel with increased spleen cell content of cyclic AMP (*41*). Although such experiments do not establish which functional cell types were involved either in immune suppression or in its prevention by drugs, they demonstrate a clear-cut effect of cyclic AMP on the initial stage of a humoral antibody response and provide a model for investigation of its mechanism. This mechanism could involve either antigen recognition or a cell analogous to the "regulator" cell suggested by the experiments with the hormone-Sepharose columns described above.

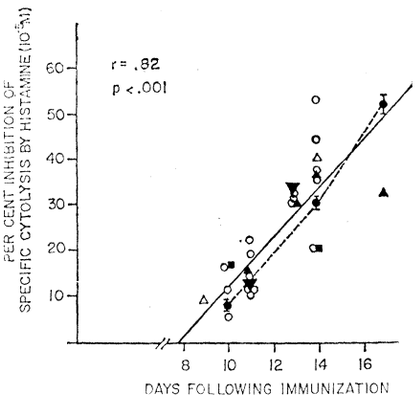

Fig. 2. Adult C57BL/6 mice were immunized intraperitoneally with 10^7 DBA/2 mastocytoma cells. The cytolytic activity of spleen cell suspensions was assessed at various times thereafter, and the inhibition of cytolysis by $10^{-5}M$ histamine was measured on each occasion. Data from 30 experiments are shown, together with the linear regression line for all the data. The open circles represent experiments where animals were tested on 1 day only; in these cases, lymphocyte pools from three to ten animals were used. The other symbols represent experiments where different animals from simultaneously immunized groups were sequentially followed; in these cases pools of three to five animals were used. In the group of animals represented by the symbol ● - - - ●, the mean percentage inhibition of cytolysis due to $10^{-5}M$ histamine together with the S.E.M. of triplicate assays are shown for 10, 14, and 17 days after immunization. [From (*28*); courtesy of *Nature (Lond.)*]

Several laboratories have attempted to study the effect of cyclic AMP on proliferation of lymphocytes induced by antigen (for example, tuberculin) or several plant mitogens, such as phytohemagglutinin (PHA). The proliferation, characterized by transformation of lymphocytes into large, blast-like cells that rapidly synthesize protein, RNA, and DNA, has been thought of as an in vitro model of the antigen-induced proliferation of a clone of specifically immunocompetent cells. Such proliferation, a prerequisite for the mounting of an effective immune response, may be thought of as an "amplification" process. The PHA-induced transformation of human lymphocytes in vitro is inhibited by the entire range of cyclic AMP–active agents that inhibit immunologic effector mechanisms, including prostaglandins and β-adrenergic catecholamines (*42*). This suggests that cyclic AMP may inhibit amplification of an immune response, in parallel with inhibition of the effector mechanisms outlined above.

These issues can best be explored in recently developed elegant experimental systems which permit investigation of humoral or cell-mediated immune responses initiated and maintained in vitro (*43*). For example, dibutyryl cyclic AMP has been found to prevent the induction of humoral antibody responses to heterologous erythrocyte antigens in spleen cultures; the nucleotide appeared to inhibit both T and B lymphocyte function in this system (*44*).

Interestingly, guanosine 3′,5′-monophosphate (cyclic GMP) was shown to reverse the inhibitory effect of the cyclic AMP analog on induction of the immune response in the same system. In addition, cyclic GMP appeared able to replace T cells in supporting the immune response of B cells (*44*). Cyclic GMP, the only other cyclic nucleotide found in nature (*3*), is beginning to be investigated as a regulator of leukocyte function. The early reports (*45*) suggest that cyclic GMP and cyclic AMP may act in a "push-pull" fashion to regulate functions of lymphocytes, neutrophils, and even mast cells: wherever cyclic AMP appears to inhibit a reaction, cyclic GMP may enhance it; similarly, wherever sympathetic neurohormones (catecholamines) appear to

act through cyclic AMP, the parasympathetic neurotransmitter, acetylcholine (or its congeners), may act through cyclic GMP. While none of these suggestions can be considered conclusive (*45*), it is possible that the hypothesis we develop here may eventually have to be expanded to include cyclic GMP.

Constructing the Hypothesis

We have described the details of the experimental underpinnings of our general hypothesis, including (i) inhibition by cyclic AMP of a wide spectrum of inflammatory and immunologic events studied in vitro (Table 1); (ii) the consistent panoply of endogenous hormones which control function of immunocytes and inflammatory cells by stimulating cyclic AMP accumulation (Table 1); and (iii) the apparent nonrandom distribution of hormone receptors among lymphocytes, which may develop (or become expressed) during the antigen-stimulated differentiation of B and T lymphocytes (*46*). The hypothesis is based on the supposition that some or all of these in vitro observations reflect events that occur in vivo. Thus, the hormones identified by test tube experiments act in vivo on neutrophils, mast cells, and basophils to limit the intensity and extent of inflammatory, allergic, or anaphylactic reactions, and to modify immunocyte function at several stages, from the recognition of an antigenic signal, through its amplification by clonal proliferation, to the expression of an immune response by differentiated T and B effector lymphocytes.

An essential feature of the hypothesis concerns the nature of the hormones themselves, which originate either from neuroendocrine cells (the catecholamines) or from inflammatory reactions. In the first case the catecholamines provide a neuroendocrine mechanism allowing nonantigenic environmental influences to modify inflammatory or immune responses. In the latter case, the inflammatory mediators would be produced by reaction to tissue injury or by immunologic reactions per se, such as anaphylaxis; thus, the intensity or extent of a response to a specific stimulus could regulate subsequent responses to continued or repeated stimuli—the essential feature of feedback circuits or servomechanisms.

Analogous control systems have been well documented in almost every field of physiology, perhaps most prominently in cardiovascular and endocrine regulation. The existence of similar control systems in immunology is supported by real, if somewhat fragmentary, evidence. In the concluding sections of this article we adduce some of this evidence and suggest experiments designed to detect and analyze hormonal and cyclic nucleotide regulation (i) of immediate hypersensitivity and inflammation and (ii) of immunological responses.

Immediate Hypersensitivity and Inflammation

One obvious link between effects in vitro and in vivo of hormones and cyclic AMP lies in the modern treatment of disorders of immediate hypersensitivity. Antigen-induced IgE-mediated release of histamine, SRS-A, and other substances plays an important pathogenic role in some cases of asthma and in cutaneous or generalized anaphylaxis in man (*47*). Theophylline and epinephrine, two agents used successfully in the treatment of these disorders, are also good inhibitors of histamine and SRS-A release in vitro (*6, 8, 13*). In fact, sublingually administered isoproterenol, a "pure" β-adrenergic agent, prevented the cutaneous wheal-and-flare response caused by antigen—but not that caused by injected histamine—in allergic human subjects (*48*). Similar findings have been reported for systemically administered isoproterenol, PGE_1, theophylline, and histamine itself in rabbits (*49*) and monkeys (*50*). These experiments suggest that cutaneous mast cells, the probable source of the vasoactive mediators of wheal-and-flare reactions, respond to cyclic AMP–active agents very much as do basophils in peripheral blood.

Thus the results of test tube experiments accurately predict the effect of drugs on an immediate hypersensitivity reaction in vivo. Do the test tube results similarly predict regulatory effects of any endogenously liberated hormones or mediators of inflammation, such as catecholamines, histamine, and prostaglandins, as suggested in the speculative scenario of Fig. 3? If so, the experimental evidence for any particular compound in vivo should satisfy three criteria: (i) the compound itself should be present and measurable in the tissue at the appropriate time, in appropriate concentrations; (ii) the exogenously administered compound should produce the predicted results; (iii) "removal" of the compound in question, either by preventing its production or by blocking its effect (with pharmacologic antagonists), should predictably alter the overall process—in this case, the immediate hypersensitivity reaction.

One kind of experiment in vivo, for example, could take its cue from the experiments of Walker (*51*) in human lung tissue. She measured the antigenic, IgE-mediated release of prostaglandins, histamine, and SRS-A from passively sensitized fragments of human lung tissue. Indomethacin, an acidic anti-inflammatory agent which blocks prostaglandin synthesis in many tissues, abolished antigenic release of prostaglandins, and partially prevented the release of histamine; the release of SRS-A, however, was doubled. Since exogenous prostaglandins do block SRS-A release from human lung fragments (*52*), one possible interpretation of Walker's data is that the indomethacin-induced blockade of prostaglandin synthesis removed an inhibitory influence on SRS-A release. By implication, then, endogenous production of one mediator of inflammation could modulate the release of another.

Appropriate in vivo model systems are available. Chiesa and his colleagues, for example, have demonstrated histamine release into pulmonary venous blood during antigen-induced bronchoconstriction in the dog (*53*). The effect of sympathetic stimulation on histamine release should also be tested.

Often the first evidence for the importance of a particular compound in physiologic regulation comes from study of a disease state in which such regulation is deficient. Considerable recent experimentation suggests that asthma may be such an experiment of nature: specifically, that deficiency of β-adrenergic receptor responsiveness may be pathogenic in asthma (*54*). The evidence is suggestive, although not conclusive: (i) Propranolol, a β-adrenergic antagonist, can provoke severe bronchoconstriction in asthmatic subjects; (ii) both the hyperglycemia and the urinary excretion of cyclic AMP caused by catecholamines have been reported to be decreased in asthmatic subjects, as compared with normal controls (*54*). Parker and his co-workers (*54*) have reported that β-adrenergic catecholamines cause statistically less accumulation of cyclic AMP in leukocytes of asthmatics than in those of control subjects. These findings, even

if confirmed in other laboratories, fail to specify precisely how deficient β-adrenergic responses cause asthmatic bronchoconstriction. It would be exceedingly difficult to prove that bronchial smooth muscle in asthmatics is specifically hyposensitive to β-adrenergic stimuli.

In the context of the present review, another set of possibilities is equally interesting: If leukocytes or mast cells of asthmatics accumulate lesser amounts of cyclic AMP in response to any endogenous hormone than do the same cells in normal subjects, physiologic control of the release of inflammatory mediators might be deranged. Certainly such a possibility is worth investigating in asthma and other allergic disorders.

In parallel fashion, the contribution of neutrophils to an acute inflammatory response may be controlled by hormones and inflammatory mediators, although evidence for or against this possibility is only fragmentary. The β-adrenergic drugs, for example, inhibit dextran-induced paw edema in an in vivo model of the acute inflammatory response which may be mediated by neutrophils (*55*). The obvious similarity between hormonal inhibition of histamine release from basophils or mast cells and of release of lysosomal contents from neutrophils prompts the inclusion of the latter cells (Fig. 3), if only for the purpose of pointing out the need for more critical experiments in vivo.

Hormonal Messages and the Immune Response

The relevance of our general hypothesis to regulation of immune responses is sketched in Fig. 4, which incorporates much of the data cited above (see Table 1); this speculative scheme indicates (heavy wave symbols) the sites at which hormones or cyclic AMP may inhibit or regulate different stages of the immune response: the initial contact of small lymphocytes with antigen, the subsequent proliferation and differentiation of immunocytes, and the functions of differentiated effector cells. The diagram also incorporates the suggestion (*28, 31–35*) that hormone receptors may be expressed on effector cells as part of their antigen-induced differentiation.

The purpose of presenting such a scheme is to stimulate investigation of the possible roles of vasoactive hormones and cyclic AMP at all stages of immunologic regulation. The scheme points up several groups of questions which appear worthy of further investigation:

1) Histamine and the prostaglandins, messengers which consistently affect most in vitro immunologic models in which they have been studied, are also well known as mediators of inflammation. If this is not merely coincidence, what does it mean? Is it possible, for example, that antigen-induced release of histamine from mast cells acts not only to constrict bronchi or to produce a cutaneous wheal, but also to limit the extent of such reactions by preventing further release of mediators from cells at the periphery of an immediate hypersensitivity reaction (see Fig. 3)? Similarly, do the tissue injury and resulting inflammation that often accompany an antigenic challenge affect the subsequent immunologic response by changing the cyclic AMP content of the immunocytes destined to respond to the antigen? Such questions would have to be approached with careful experiments, exploiting the availability of new, potentially specific pharmacologic antagonists and controlling the conditions under which an animal's immunocytes first come in contact with antigen.

2) The complex interactions of immunocytes with any antigen initiate a group of very important decisions, which determine the direction of the resulting immunologic response: immunoglobulin production (which class of immunoglobulin?) as compared to cell-mediated (delayed) hypersensitivity, immunologic tolerance as compared

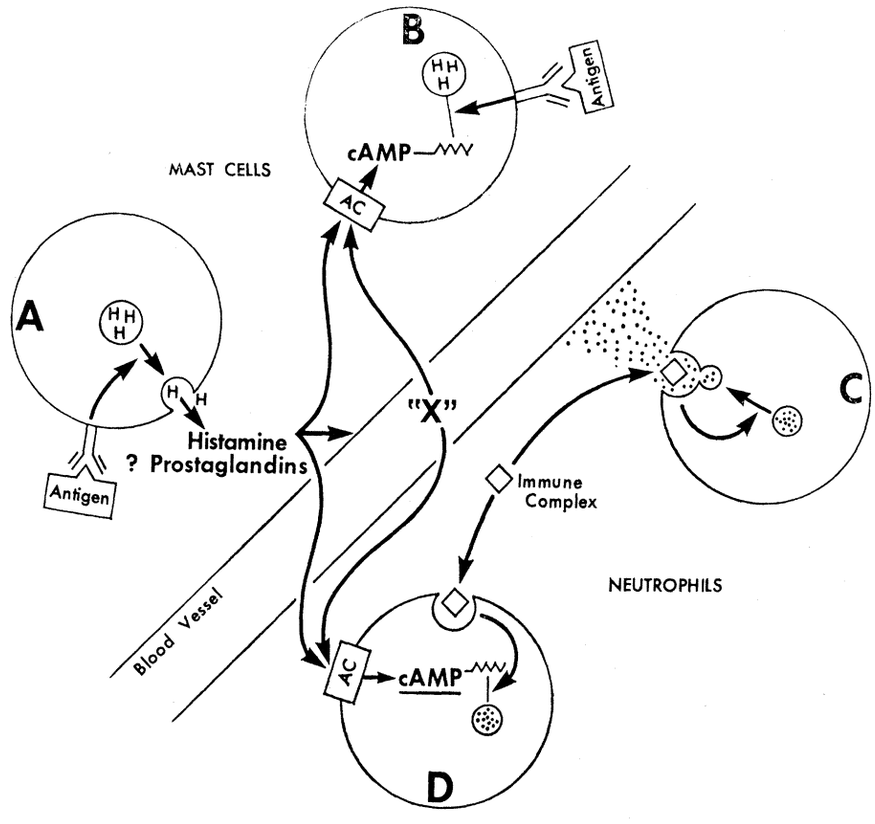

Fig. 3. Speculative scheme illustrating the possibility that release of inflammatory mediators such as histamine or prostaglandins might limit the extent or severity of an inflammatory reaction. Combination of antigen with IgE on the membrane of mast cell A causes release of histamine and other mediators (prostaglandins?). Similarly, immune complexes of antigen and antibody can be phagocytized by neutrophils (C, right) and result in release of a portion of the neutrophil's lysosomal contents into the extracellular medium. Both the inflammatory "hormones" and the lysosomal contents presumably act on blood vessels as mediators of acute inflammation. It is possible, however, that the hormones (and perhaps even some neutrophil lysosomal component, not shown) stimulate adenylate cyclase (*AC*) in other cells (mast cell B, neutrophil D) to inhibit release of intracellular mediators which would otherwise extend or intensify the inflammatory reaction. It is also possible that other endogenous anti-inflammatory factors, designated "*X*", blood-borne or released by sympathetic nerves (for example, catecholamines), act through cyclic AMP (*cAMP*) in a similar fashion to regulate an inflammatory reaction.

to an actively maintained response, and the like. We know that many of these decisions require interaction between different kinds of cells—for example, macrophages, T lymphocytes, and B lymphocytes. Are any of these interactions mediated or regulated by cyclic AMP? If so, what are the "first messengers"? Such questions can be approached in model systems (*43*) which allow dissection of the various cell types contributing to an immune response, and their pharmacologic manipulation.

3) What is the physiologic meaning of the apparent development of certain hormone receptors in the course of differentiation of T or B lymphocytes stimulated by specific antigen? The implied absence of such receptors in undifferentiated immunocytes, prior to transformation by antigen, suggests that other messengers would be required to control their function.

4) If a "regulator-suppressor" cell which binds to insolubilized hormone-carrier columns exists, it should be isolated and characterized. What actual messages does it receive by virtue of hormone receptors on its surface? What message does it emit to exert control over proliferation of antibody-forming cells? Do the same or similar cells control other types of immune responses, such as delayed hypersensitivity?

5) Why should lymphocytes, or indeed neutrophils or mast cells, possess β-adrenergic receptors? Does this mean that the sympathetic nervous system and the adrenal medulla in fact exert control over immunologic responses? In some respects such questions have their counterparts—also still unanswered—in the multifarious effects of adrenal glucocorticoids on immune responses. These effects have been exploited for treatment of immunologic diseases, but their physiologic meaning is far from clear. It should prove possible to investigate adrenergic influences on immunologic responses, with the use of the already highly developed methodology for investigation of adrenergic control of the cardiovascular system. This methodology includes the possibility of specifically inhibiting or stimulating sympathetic reflexes, sensitive and accurate measurement of the catecholamines themselves, and, of course, methods applicable to cyclic AMP itself, an important mediator of many adrenergic effects (*3*).

This review has emphasized the test tube origin of most of the observations which led to our general hypothesis. Application of these findings to the vastly more complex events that result in an immune response in whole animals will certainly not be simple. For example, Braun's investigations of effects of agents like isoproterenol and theophylline on humoral immunity in vivo produced an array of apparent stimulatory and inhibitory effects (*56*). When cholera enterotoxin, a stimulator of adenylate cyclase in lymphocytes (*12*, *26*), was administered to mice after immunization with allogeneic cells,

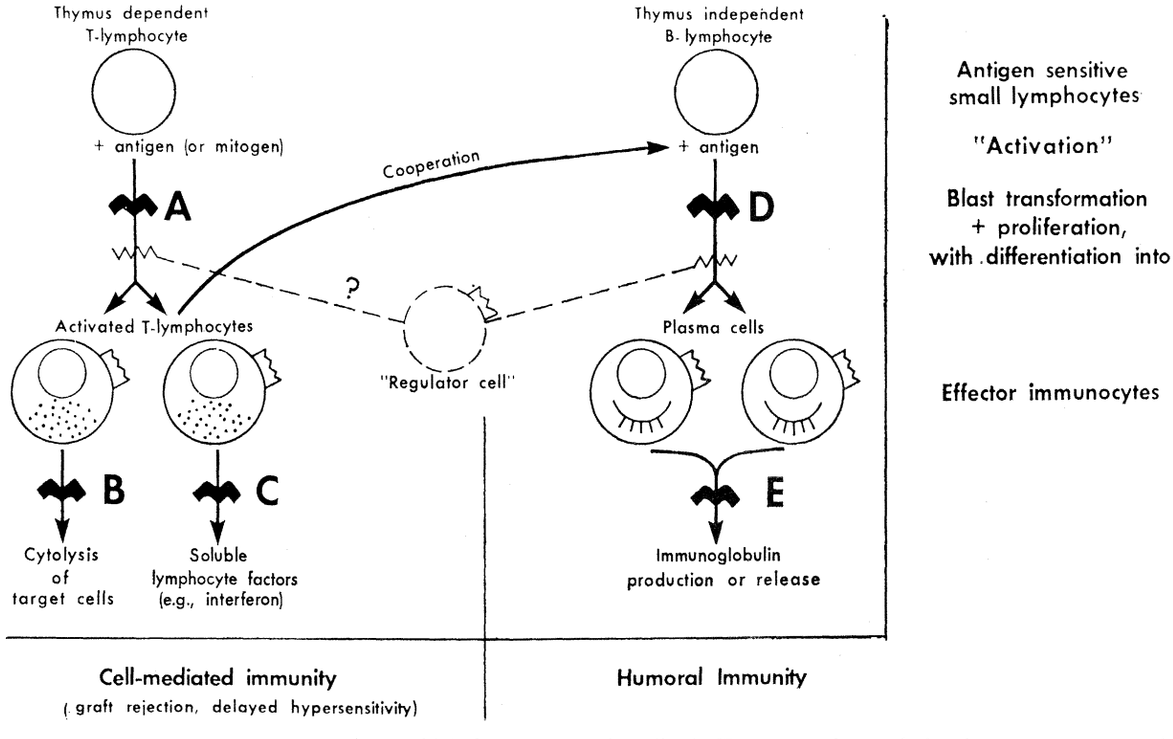

Fig. 4. Simplified scheme illustrating sites at which hormones and cyclic AMP may act in regulating immune responses. Antigen-specific thymus-dependent (T) and thymus-independent (B) lymphocytes are thought to be "activated" by contact with antigen to produce either specific tolerance (not shown) or blast transformation, followed by proliferation of differentiated clones of effector immunocytes capable of producing antibody- or cell-mediated immunity. The sawtooth symbols on the surface of effector cells indicate that the appearance of hormone receptors may be one result of their differentiation. The heavy wave symbols indicate steps at which experimental evidence suggests that hormones (prostaglandins, histamine, catecholamines) or cyclic AMP exerts inhibitory effects in vitro. These steps are discussed in more detail in the text: (A) Inhibition of mitogen- or antigen-induced blast transformation and proliferation of T lymphocytes (*42*). (B) Inhibition of specific immune cytolysis of allogeneic target cells (*12*, *16*, *28*). (C) Inhibition of production or release of soluble lymphocyte products such as interferon (*19*). (D) Reversal of antigen-induced immune suppression when lymphocytes are exposed to cyclic AMP–active drugs during initial contact with antigen (*40*, *41*), prevention of humoral (agglutinating) antibody response to allogeneic cells by cholera enterotoxin (*58*), or inhibition of proliferation of antibody-forming cells (*44*). (E) Inhibition of production (or release) of antibody to heterologous erythrocytes (*23*). A hypothetical "regulator cell," detected by its binding affinity for insolubilized histamine conjugates (*32*), is also shown, although its relation to the cyclic AMP systems is not defined.

the immune response was suppressed. The appearance of specific cytolytically active T lymphocytes was prevented, and agglutinating antibodies in the serum were markedly suppressed (*57*). In these experiments the toxin did not cause prolongation of allograft survival (*57*), although Warren (*58*), using different mouse strains, has subsequently shown that the toxin could significantly increase allograft survival. Such experiments cannot be interpreted without careful qualification. The most obvious problem is the potential lack of specificity of the toxin itself, which stimulates cyclic AMP accumulation in most cells (*11*), and may have other effects not related to cyclic AMP. Such experiments, however, represent early attempts at investigating a potentially important mechanism for regulation of immunity. Further understanding of such a regulatory mechanism may open the way to new therapeutic approaches to treatment of immunologic and inflammatory disorders in man.

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Amine conjugates were made either by linking the free amino group of the amine in a peptide bond with a carboxyl group of the carrier protein or peptide (with carbodiimide as the coupling reagent) or by converting the primary amine function to a secondary amine via a glutaraldehyde linkage (*30*). The carriers or conjugates were linked to Sepharose (agarose) beads activated by cyanogen bromide [K. L. Melmon, H. R. Bourne, Y. Weinstein, M. Sela, *Science* **177**, 707 (1972)]; K. L. Melmon, Y. Weinstein, G. M. Shearer, H. R. Bourne, S. Bauminger (*31*); K. L. Melmon, Y. Weinstein, G. M. Shearer, H. R. Bourne, in *Cyclic Nucleotides, Immune Responses and Tumor Growth*, W. Braun, C. C. Parker, L. M. Lichtenstein, Eds. (Academic Press, New York, in press).

Diphenhydramine, a histamine antagonist, displaced cells only from histamine conjugates; propanolol (a β-adrenergic blocking agent) displaced cells only from the conjugates of norepinephrine in which the amine group was converted to a secondary amine. This conversion of the primary to the secondary amine resulted in a catecholamine resembling epinephrine rather than norepinephrine. The α-adrenergic blocking agents had no effect on the binding. High concentrations of isoproterenol and epinephrine displaced cells from conjugates of the catecholamine. Histamine, but not the catecholamines or prostaglandins, displaced cells from the insolubilized histamine preparations [Y. Weinstein, K. L. Melmon, H. R. Bourne, M. Sela, *J. Clin. Invest.* **52**, 1349 (1973)].

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Experiments with a different method for detecting amine receptors (Y. Weinstein, S. Segal, K. L. Melmon, unpublished) produced evidence supporting this proposition. Soluble hormone-carrier conjugates labeled with fluorescein were prepared by methods similar to those described in (*31*), allowing the detection of binding of the hormone-conjugate to cells by fluorescence microscopy. Histamine-RSA-fluorescein was found to bind to about 8 percent of splenic leukocytes from unimmunized mice, while RSA-fluorescein bound to less than 1 percent of the cells. Histamine (but not catecholamines) and antihistamines (but not antiadrenergic agents) displaced the hormone-conjugate from the cells. Further, when the spleen cells were taken from mice immunized with SRBC, the proportion of cells binding histamine-RSA-fluorescein was increased severalfold as compared with binding to cells from unimmunized litter mates. These experiments indicate that the hormone and not the carrier critically contributes to the binding of hormone-conjugates by cells, and that mouse spleen cells develop or express receptors for the hormone-conjugate during the process of immunization.

One obvious alternative explanation must be considered. Passage of the cells over hormone-conjugate columns could have depleted any antibody-producing cells present in the donor spleen cell suspension, thereby removing a potential source of antibody-mediated feedback control [J. W. Uhr and G. Moller, *Adv. Immunol.* **8**, 81 (1968)] of the response generated by the transferred cells. This explanation appears unlikely, because (i) the spleens of unimmunized donor mice contained very few antibody-forming cells and (ii) an enhanced recipient antibody-forming cell response was obtained in all three mouse strains tested, although plaque-forming cells from one of the mouse strains (C57BL/6) were not retained by the columns (*31, 32*).

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Several assay systems for this type of experimentation exist. Th R. D. Mishell and R. W. Dutton [*J. Exp. Med.* **126**, 423 (1967)] and J. Marbrook [*Lancet* **1967-II**, 1279 (1967)] have independently demonstrated antibody induction to sheep erythrocytes in vitro; and, more recently, H. Ginsburg [*Immunology* **14**, 621 (1968)], G. Berke, G. Yagil, H. Ginsburg, M. Feldman [*ibid.* **17**, 723 (1969)], and H. Wagner [*Eur. J. Immunol.* **1**, 498 (1971)], and their co-workers have demonstrated the in vitro induction of cytolytically active T lymphocytes. Thus, in vitro systems exist for the induction of both cell-mediated and humoral immune responses.

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46. This last point, if true, may prove important for the construction of a general hypothesis, since receptors could be present on lymphocytes simply because they are proteins coded in the genome of all cells; the receptors could be expressed only because their presence confers no selective disadvantage to the organism: This would be the case if the lymphocyte never "saw" prostaglandins, histamine, or catecholamines in high enough concentration to affect its function. Nonrandom distribution of receptors, then, suggests that they may perform a physiologic function.
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Enzyme Polymorphism and Metabolism

Polymorphism among enzyme loci is related to metabolic function.

George B. Johnson

Since the time of Darwin and Wallace, there have been arguments concerning the evolutionary significance of patterns of natural variation. Evolutionary biologists are now involved in a controversy over the question of whether or not genetic polymorphisms at enzyme loci are maintained by selection. Sufficient experimental data now exist to indicate that they are and to suggest their role in evolutionary processes.

Polymorphism and Selection

The current controversy over the selective significance of enzyme polymorphism has roots extending back several decades in the history of population genetics to the arguments of Fisher and Wright concerning the significance of genetic drift (*1*). Now, however, the same issues are being argued in a different context. In the absence of experimental data on the amounts of genic variation being maintained in natural populations, Kimura and Crow (*2*) suggested from theoretical considerations that the maintenance of variation should entail an evolutionary cost, or "genetic load," and that because of this, the total amount of polymorphism in natural populations may not be great; excessive genetic load would be expected to result in population extinction (*3*). However, with the advent of electrophoresis as a common tool for surveying genetic variation of enzyme loci (*4*), it has become apparent that the amount of polymorphic variation at the enzyme loci of natural populations is quite high (*5–14*), far higher than could exist if the original genetic load concepts were correct. In view of these results, such concepts could be maintained most simply by assuming that no selectively important differences exist among the electrophoretic variants. Thus, the argument has been advanced (*15*) that the variant proteins contain only minor differences in tertiary structure which are sufficient to affect electrophoretic mobility but not to affect significantly the functioning of the enzyme. Because electrophoretically different alleles are seen as functionally identical, they are thought to affect the organism's fitness identically, the differences among them thus being neutral to the action of selection.

Experimental evidence is now becoming available (*16*) which permits a test of the hypothesis that enzyme polymorphisms are selectively neutral. Most reported surveys of electrophoretic variation in natural populations have provided evidence of nonrandom processes. Biogeographic patterns are often reported to be uniform over wide geographic ranges (*11, 12, 17*), or to reflect parallel patterns of environmental variation (*18–22*). Analysis of the allele frequencies reported in these studies also reveals nonrandom processes (*23*), although confusion may arise when data obtained from diverse organisms are pooled (*24*). The force of such arguments is difficult to evaluate, however, because of uncertainty about the possible involvement of migration (*25*), linkage (*26*), and founder effect (*27*). Neither is it clear when data concerning individual loci should be regarded as special cases and when they may be considered as illustrating a more general principle; those individual cases where selection is implicated at an allozyme locus do not necessarily argue powerfully for the generality of selective significance. It seems clear, however, that while some caution is necessary in evaluating the diverse array of information, much of the evidence is against Kimura's hypothesis.

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