Regulation of Immunity and Inflammation by Mediators From Macrophages

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Mononuclear phagocytes secrete a number of materials into the extracellular environment. The materials secreted by phagocytes can be grouped into three categories: a) enzymes affecting extracellular proteins (collagenase, elastase, lysosomal proteases, plasminogen activators), b) materials involved in defense processes (complement proteins, interferons, lysozyme), and c) factors regulating activities of surrounding cells. The latter include lymphostimulatory molecules, a colony-stimulating factor, and inhibitors of cell growth. The conditions for secretion of the materials depend on the activity of the phagocytes. The lymphostimulatory molecules secreted by macrophages exert various effects: 1) an increase in DNA synthesis of lymphocytes, 2) a maturation of early thymocytes to mature T cells, and 3) the differentiation of some B cells to antibody-secreting cells. The mitogenic principle has been partially isolated as a protein of 15,000 to 20,000 daltons. The secretion of lymphostimulatory molecules is increased following uptake of various materials by macrophages or by addition of activated T cells to macrophage cultures. (Am J Pathol 85:465–478, 1976)

THE MONONUCLEAR PHAGOCYTES are cardinal cellular elements in host resistance and in inflammation. The monocyte-macrophage system of cells is actively involved in phagocytosis and intracellular digestion. These cells participate in an essential way in the elimination of various kinds of microorganisms as well as in the elimination of debris, dead cells, and tissue during the cleaning of wounds. Phagocytes have a fundamental role in specific immunity through an intimate functional association that has developed between them and the lymphocyte, principally those of the thymic class. Phagocytes participate in early inductive events favoring in some way or another the early interaction of T and B lymphocytes with antigen. In cellular immunity the phagocytes are the main effector cells, the lymphocytes representing the specific recognition limb of the reaction.

The precise ways by which phagocytes carry out their many functions are not clear. In this paper, we consider the secretory function and raise the point, also discussed by others,[1,2] of its potential biologic importance. It is clear that the mononuclear phagocytes release a number of active

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products into the extracellular environment in a truly secretory process. Through this secretory function, which can be extensive, the phagocytes can modulate the activity of surrounding cells and/or tissues. As a result of the intensive work in several laboratories during the past few years, we have substantial information on the various secreted materials and, more importantly, on the conditions for increased secretion. The secretory function of mononuclear phagocytes has been reviewed recently in detail.[3] In this article, we summarize the most significant features of this function and highlight the secretion of lymphostimulatory molecules, which has been the subject of our own research interest.[4-7]

Products Secreted by Phagocytes

Table 1 summarizes the various products found in cultures of phagocytes. Among the secreted products are enzymes including lysosomal enzymes and others like lysozyme, plasminogen activator, collagenase, etc.; also secreted are complement proteins, interferons, and various biologically active materials. Among the biologically active products are included the colony-stimulating factors, the lymphostimulatory factors, and various inhibitory or cytolytic molecules. As noted in Table 1, the secreted products can be grouped into three interrelated categories: a) enzymes that affect extracellular proteins, b) products involved in host defense, and c) products affecting surrounding cells. All of these secretory products have been identified in culture fluids from phagocytes obtained either from peripheral blood as monocytes or from the peritoneal cavity, usually of mice or guinea pigs, as macrophages. A brief comment on the various secretory products follows. (See Unanue [3] for a comprehensive reference list on this subject.)

Part of the content of lysosomal enzymes is released into the medium during the interaction of phagocytes with various materials.[1,8-12] Release takes place during phagocytosis of zymosan or polystyrene particles, bacteria or bacterial cell walls, antibody–antibody complexes, etc. The release of lysosomal enzyme has also been studied in fibroblasts[13] and neutrophils (reviewed by Becker and Henson [14]). In the neutrophil the release takes place, as in the macrophage, during phagocytosis or as a result of contact with surfaces coated with antigen–antibody complexes. In the former case, part of the release occurs when the lysosome fuses with an endocytic vesicle that has not yet closed, maintaining a communication via a stoma to the outside. In the latter case, the release is attributed to migration of the lysosomal vesicles to the stimulated surface membranes in a process resembling endocytosis, but in reverse.

In both cases, the release of lysosomal enzymes is selective—a cyto-

Table 1—Products Secreted by Phagocytes

Enzymes affecting extracellular proteins
Lysosomal enzymes
Plasminogen activator
Collagenase
Elastase
Products involved in host defense
Lysozyme
Complement proteins
Interferon
Microbicidal products
Products modulating cells
Colony-stimulating factors
Lymphostimulatory proteins
Low-molecular-weight inhibitors

plasmic enzyme like lactate dehydrogenase is not secreted or released unless the cell is killed. The amount of lysosomal enzymes released upon brief contact with a large phagocytic stimuli is usually about 10 to 25% of the total enzyme contents, that is, only a relatively small portion. This release usually takes place in a question of minutes. There are two important features concerning lysosomal enzyme release from macrophages. First, under certain circumstances, in contrast to the neutrophil, it can be quite extensive. This point became evident through the work of Davies *et al.*, in which macrophages challenged with streptococcal cell wall preparations become "activated," releasing, after a few days, up to 80% of their content of lysosomes.[11] Thus, an activated macrophage phagocytizing large, partially insoluble materials releases, continuously, amounts of enzymes which could affect the structure of extracellular proteins. Davies *et al.* hypothesized that such extensive release could account for the inflammatory reaction seen when the streptococcal cell walls are injected into animals.

A second important feature of lysosomal enzyme release in macrophages is that it can be stimulated by products from activated lymphocytes.[15] Thus, the process is stimulated not only by contact of the macrophage membrane with an insoluble particle but also by interaction with soluble active materials.

Besides lysosomal enzymes, phagocytes secrete a number of other enzymes which include lysozyme,[16-20] plasminogen activators,[21,22] collagenase,[23,24] and elastase.[25]

Lysozyme, a protein of about 14,000 daltons, is found in abundance in body fluids. It acts on the peptidoglycan of certain bacterial cell walls, specifically hydrolyzing the one to four glycosidic linkages between acetylmuramic acid and acetylglucosamine. Plasminogen activators con-

vert plasminogen to plasmin, resulting in fibrinolytic activity. Macrophages secrete two molecular species of 48,000 and 28,000 daltons.[21] The macrophage collagenase is a metalloprotein of the same specificity as other collagenases, acting at neutral pH by splitting tropocollagen into fragments of one-fourth to three-fourths of the original chain length.[24] The elastase from macrophages is a serine protease which degrades insoluble elastin. It has properties different from pancreatic and granulocyte elastase.[25]

The secretion of these four enzymes differs somewhat from that of lysosomal enzymes. Secretion of lysosomal enzyme is usually limited to part of their content; usually the majority of the enzymes remain inside the cell to function in intracellular digestion. This is not the case for the others which are synthesized for export and are not retained by the cell. However, within this group, the characteristics of secretion differ quite markedly, a clear indication of the complexity of the secretory process. Gordon *et al.* found that macrophages secreted, continuously, about 80 to 90% of their content of lysozyme regardless of whether or not the cells were stimulated to phagocytize.[19] Activated macrophages secreted larger amounts of lysozyme.[18] In contrast, the other three enzymes were secreted mostly following phagocytosis. Two other important points need to be highlighted: a) the extent of secretion of plasminogen activator following phagocytosis depended upon the previous "activity" of the macrophage.[22] For example, macrophages previously activated by endotoxin secreted much larger amounts of the enzymes upon subsequent challenge than nonactivated cells. b) The nature of the phagocytic stimuli conditioned the time course of release; while digestible antigen–antibody complexes triggered a burst of secretion, indigestible latex beads stimulated persistent secretion for days and days of culture.[22]

It is well established that phagocytes make some of the proteins of the complement system.[26-30] Phagocytes synthesize and release C2, C4, C3, and most likely C5. The release of C4 and C2 increases following phagocytosis of bacteria but not of latex particles.[29]

Also secreted in macrophage cultures are interferons[31-33] and a pyrogenic material[34-36] as well as a colony-stimulating factor. The colony-stimulating factor is necessary for the formation of hematopoietic colonies from stem cells.[37-41] Exposure of macrophages to endotoxin[40] or to synthetic polynucleotides results in increase in colony-stimulating factor.[42]

The Lymphostimulatory Molecules

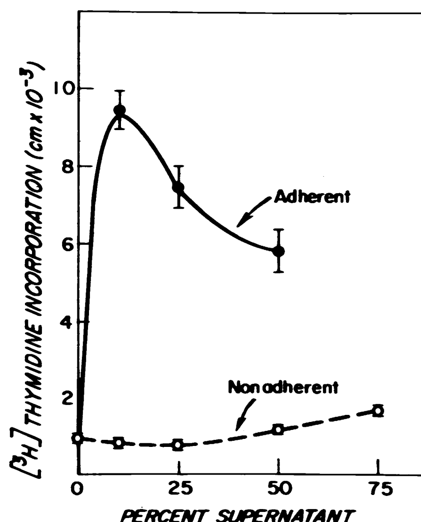
Our purpose here is to summarize the main results of our own research in this field.[4-7] Others have also reported on similar results,[43-49] but these

will be referred to only in passing. Most of the observations reported here have been published or are in press.

We have found that medium in which macrophages have been cultured for 6 to 24 hours can exert marked biologic effects on lymphocytes *in vitro*. The lymphostimulatory medium is first dialyzed to remove low-molecular-weight components which inhibit cell division (described later in this section).[6] The effects of the conditioned medium on the proliferation or differentiation of lymphocytes can then be assayed.

Several biologic activities can be identified in macrophage culture medium. One consists of a mitogenic principle which stimulates thymocytes preferentially; peripheral B and T lymphocytes show rather limited enhancement of ^{3}H-thymidine incorporation (Text-figure 1). Gery *et al.*[43,44] and others have described a lymphocyte-activating factor (LAF) which allows thymocytes to respond to lectins such as phytohemagglutinin (PHA) which normally stimulates only mature T cells. It is plausible that our mitogenic principle is similar to LAF, although we find that the macrophage culture medium not only enhances the response to thymocytes to con A and PHA but is strongly mitogenic by itself.

The effects of macrophage secretions on thymocyte maturation have been studied by one of us (DIB).[7] Immature thymocytes cultured with samples of the conditioned medium for 24 to 48 hours change their content of H-2 antigens from a low level to the high level characteristic of the cortisone-resistant, mature thymocyte. At the same time, the thymocyte responds much more effectively in a mixed lymphocyte reaction (MLR). This mature phenotype is stable, since the MLR and response to



TEXT-FIGURE 1—Medium obtain from 24-hour cultures of dish-adherent (i.e., macrophage-rich) and nonadherent (i.e., lymphocyte-rich) spleen cells was used to culture thymocytes. Thymocytes were cultured for 3 days at various concentrations of the conditioned medium.

lectins have been assayed up to 4 days after the macrophage-conditioned medium has been removed and replaced with fresh culture medium.

The increase in amount of H-2 and sensitivity to the MLR reflects true maturation of the immature thymocyte. That it is not the result of differential cell growth of the mature thymocytes already present in the thymus is demonstrated by assaying the conditioned culture medium on immature thymocytes isolated on an albumin gradient. The immature thymocytes are operationally defined as that fraction showing no significant PHA responsiveness and severely limited ability to respond in the MLR or absorb anti–H-2 antibodies.

Furthermore, inhibition of 99% of stimulated cell division by mitomycin does not appreciably affect the H-2 increase. This rules out the possibility that a few contaminating mature thymocytes could be dividing preferentially in response to the macrophage-conditioned medium. It also clearly shows that cell division is not required in this differentiation assay. It, therefore, appears that maturation follows *activation* of the thymocyte by a macrophage factor. Cell division also ensues but is not required for differentiation. This is supported by our kinetic studies which show that H-2 transition preceeds blastogenesis in these cultures. The molecule responsible for the maturation has not been identified yet.

Another biologic activity found in macrophage cultures is a B-cell maturation principle. B cells from mice primed to hapten-proteins incubated with the macrophage-conditioned medium differentiate to antibody-secreting cells. The differentiation is best seen with hapten-primed cells and is poorly seen in unprimed cells (Table 2). For differentiation to occur, the cells must be incubated in macrophage culture medium but do not require the addition of antigen to the culture.

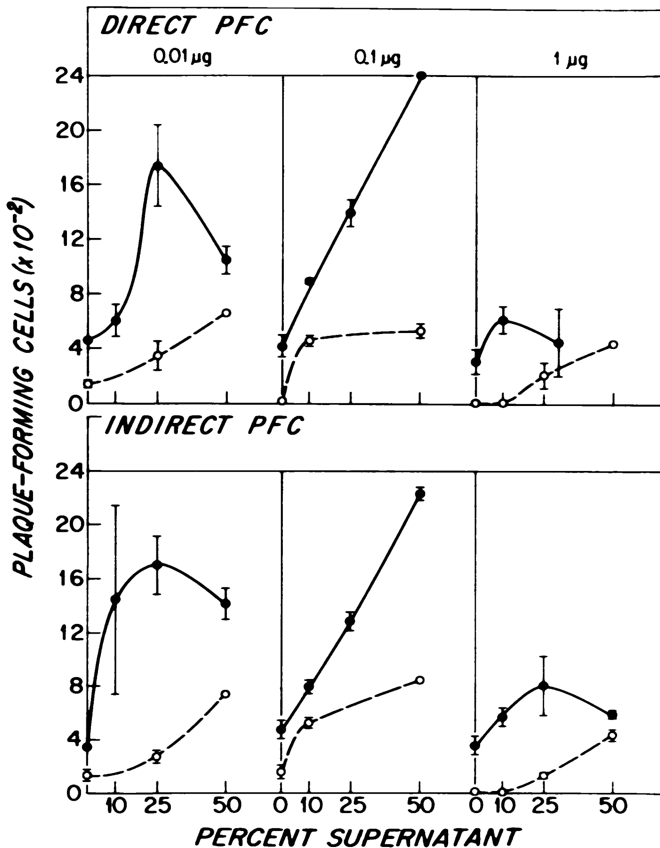
Finally, there is a marked increase in the production of antihapten-

Table 2—Differentiation of B Cells by Macrophage Culture Fluids

Method of priming	Plaque-forming cells		
	No MCF	25% MCF	50% MCF
F-KLH in complete Freund's adjuvant			
IgM	60	867	1,467
IgG	73	1,930	3,560
F-KLH in alum			
IgM	20	653	753
IgG	30	607	1,100

Spleen cells of mice immunized 30 to 45 days before, as described, were placed in culture in Mishell-Dutton type conditions for 4 days, at which time the number of antibody-forming (plaque) cells to F was determined. Cells were cultured in regular medium or in medium containing 25% and 50% macrophage culture fluids. Values are mean number of plaque-forming cells from triplicate cultures secreting IgM or IgG antibodies. Results similar to this were obtained in cultures devoid of T cells. The experiment is interpreted to indicate the capacity of the macrophage culture fluid to differentiate memory B cells.[50]

specific, antibody-secreting cells by culturing lymphocytes from hapten-protein–immunized mice in macrophage-conditioned medium. We interpret this effect as probably reflecting a combination of both principles (Text-figure 2). We have tested this effect by culturing spleen cells from mice immunized with hapten fluorescein (F) conjugated to the strong immunogenic protein hemocyanin (KLH). In such a system, production of antibody-forming cells to F depends upon collaboration of carrier-specific T cells with the hapten-specific B cells. Thus, addition to the culture of F-KLH triggers antibody formation; but this is not the case with F bound to an unrelated carrier protein-like rabbit IgG (RGG). Culture of F-KLH–primed spleen cells in macrophage fluids results in an increase in antibody-forming cells regardless of whether F-RGG is added, a reflection of the B-cell differentiating activity described above. Furthermore, addition of F-KLH together with the macrophage principles now produces a spectacular rise in antibody-secreting cells (Text-figure 2), a



TEXT-FIGURE 2—Spleen cells from mice immunized with F-KLH were cultured for 4 days with different amounts of F-KLH (*solid circles*) or F-RGG (*open circles*) in the presence or absence of macrophage culture fluids. Note that at the optimal antigen dose (0.1 μg) there is an increase in the response to both F-KLH and F-RGG in the presence of macrophage fluids.

summation of the helper T-cell activity plus the action of the macrophage principles.

It is interesting to note that there appears to be a fine balance between the stimulation produced by the macrophage principle and the T-cell helper stimulation. In our hands, the best stimulation by macrophage fluids in such a hapten-protein system was noted in cultures from mice primed for longer periods of time before culture—these cultures by themselves gave weak responses which were readily augmented by addition of the antigen plus the macrophage principle. In contrast, spleen cells from recently primed mice, which, by themselves, gave strong responses *in vitro*, behaved somewhat differently. In these instances, the degree of added stimulation by the macrophage principle in cultures challenged with the antigen F-KLH was slight. Furthermore, the dose was critical in that suppression of response could be seen with relatively low doses of the macrophage factor. We have interpreted this to mean a competition of two active moieties—that from the macrophage factor and that from the T cell—for the B cell.

Biochemical studies on the macrophage culture fluids [4,51] indicated that the mitogenic principle was a protein sensitive to chymotrypsin and papain but resistant to trypsin or to DFP. The principle passed through immunoabsorbant columns of anti-H-2 antibodies. In our first studies, fractionation on Sephadex columns indicates all activities to be in one peak corresponding to a molecular size of about 15,000 to 20,000 daltons. Subsequent studies, however, have disclosed heterogeneity with additional activities in fractions of higher molecular weights. Further studies are in progress.

The mitogenic protein found in macrophage cultures can act on B and T cells from different strains of mice and can even cross species. Factor produced from human monocytes can act on murine lymphocytes.[15] It should be noted that strains of mice differ in the extent of response to the mitogenic principle, some responding consistently at a high level, while others only at a low level. These differences are unrelated to the H-2 haplotype of the strain. C57BL/6 (an H-2^b mouse) thymocytes respond poorly to macrophage culture fluids from macrophages of the same strain or from others; A/St (an H-2^a) or BALB/c (an H-2^d) respond strongly to the factor from C57BL/6 macrophages or from syngeneic macrophages. The lymphocytes from C57BL/6 mice also responded poorly to other mitogens.

The conditions for secretion of the active principles have been under investigation. The basal production of the active principles varies greatly from preparation to preparation of macrophages, but the production changes upon stimulation. There are two situations that lead to an in-

creased secretion: a) addition to the cultures of various soluble or particulate materials that interact with the macrophages or b) addition of activated T lymphocytes.[5]

We have found that addition of antibody-coated red cells, latex particles, endotoxin, beryllium salts, or bacteria to macrophage cultures produced a five- to tenfold increase in secretion of the active principles. The production of material was rapid, reaching maximum usually at 24 hours and then declining to basal levels by 72 hours, irrespective of the nature of the stimuli. This increase was seen for all biologic activities.

A second manipulation that causes a very marked increase in the production of active principles is the addition of activated T lymphocytes to the macrophage cultures. We came across this finding when studying the activities of peritoneal exudate cells from mice infected with *Listeria monocytogenes*. These exudates produced a marked amount of active materials. In this experiment the mice were infected intraperitoneally with a sublethal dose of *Listeria* organisms, the exudates were harvested 10 days later at a time when the peritoneal cavity was clean of bacteria. Because *Listeria* immunity produces marked activation of T cells, the exudates were depleted of any possible adherent T cells by treatment with anti-θ antibodies in a cytolytic system. The very high production of active material was totally abolished by this treatment. In other experiments the T lymphocytes from the peritoneal cavity were isolated and added to normal macrophages or culture alone. While the T lymphocytes by themselves did not produce any activity, mixing them with macrophages generated very potent principles. In other experiments we have been able to show that the addition to macrophages of purified T lymphocytes from mice immunized to KLH resulted in a marked increase in activity. In these instances, however, addition of the antigen was required. The optimal T cell/macrophage ratio was about 5:1 to 10:1. We do not know which cell makes the active principles in these situations. The data is consistent with the macrophage, but this point must be further explored. In all these experiments, cultures of isolated T lymphocytes with or without antigen did not produce any activity. These experiments clearly indicate the fascinating interrelationships between the T lymphocyte and the macrophages in that only when the two were mixed did synthesis of the factors take place.

We have asked the question whether activated macrophages secrete more of the lymphostimulatory principles than the normal, nonactivated counterparts. The secretion of enzyme discussed before was markedly augmented by activation of the cells—macrophages harvested from mice injected with endotoxin or thioglycollate broth, for example, secreted

much larger amounts of lysosomal enzymes, lysozyme, plasminogen activators, collagenase, or elastase. To our surprise, activated macrophages (by endotoxin or thioglycollate) secreted very *small* amounts of the active principle under basal conditions or following phagocytosis. Thus, for secretion of the lymphostimulatory molecules, the state of macrophage activation is critical in that it appears that, after a certain state of activation, the secretion is stopped, in contrast to what happens with secretion of enzymes.

Table 3 is a summary of the above-mentioned points.

Inhibitors in Macrophage Fluids

Macrophage fluids have been found to contain low-molecular-weight inhibitors of DNA and protein synthesis. We found, in agreement with others,[52-56] an inhibition of tritiated thymidine incorporation when lymphocytes were cultured with undialyzed macrophage-conditioned media. Later, it was found that the growth of a thymic leukemia, the EL-4 line (assayed by cell counts and mitotic indices), was markedly inhibited by the macrophage culture fluids. The inhibitor of EL-4 growth was dialyzable where it could be recovered.[6] Opitz *et al.* described the presence of thymidine in macrophage fluids and interpreted some of the "inhibitory" effects reported in the literature as resulting from competition between the released thymidine and the tritiated thymidine used for assaying the DNA synthesis.[55,56] Biochemical analysis of our culture fluids has confirmed the presence of a rather large amount of a thymidine-like material.[57] Indeed, a culture of 10^7 macrophages secreted 1 μg/ml nucleotide/24 hours of culture. The effects of thymidine extend beyond a simple competition for the radioactive thymidine used in the assay for

Table 3—Summary of Lymphostimulatory Molecules

Activities
Mitogenic principle for thymocytes and, to lesser extent, for B and T lymphocytes
Rapid maturation of immature thymocytes into mature, competent thymocytes
Differentiation of memory B lymphocytes to antibody-secreting cells
Increase helper activity in hapten-protein assays
Properties of mitogenic principle
Active against various mice strains—no H-2 restriction
Not bound by anti-H-2 columns
Sensitive to chymotrypsin and papain
Resistant to DFP
About 15,000 to 20,000 daltons
Secretion
Low basal secretion in culture
Secretion increased by phagocytosis
Secretion increased by addition of activated T cells
Activated macrophages secrete less than stimulated macrophages

DNA synthesis (although this effect is clearly evident). Thymidine at high concentrations (10^{-2} to 10^{-4} M) is known to inhibit the biosynthesis of deoxycytidilic acid, thus restricting DNA synthesis. The EL-4 leukemia cells are, in fact, highly sensitive to thymidine, being inhibited by the amounts secreted by the macrophages, about 4×10^{-6} M thymidine, in a process reversed by 2-deoxycytidine. The source of the nucleotide needs studying. Opitz *et al.* gave evidence supporting a source from nuclear material phagocytosed by the macrophages. Although this is an obvious explanation, the possibility of endogenous synthesis and release has not been ruled out. Thymidine is not the only low-molecular-weight material released by macrophages. Another material, of about 600 daltons, has been found to inhibit cell growth.[54] We have found this material in small amounts affecting growth of EL-4 leukemia.

Conclusion

The monocyte macrophage system of cells comprises a highly mobile line capable of homing to places of nonimmunologic inflammation as well as forming important functional units with the lymphocytes. In this latter situation, the phagocytes appear to play a fundamentally basic role both in induction as well as in the effector side of the immune response. Phagocytes are highly capable of uptake and degradation of a number of unwanted materials; indeed, phagocytosis, the property by which these cells were identified, constitutes their fundamental *raison d'etre*. But, besides intervening in phagocytosis, the phagocytes make and secrete a large number of products, many of which have the potential to change their surrounding environment. It is likely that secretion by macrophages of a number of enzymes capable of modifying extracellular proteins must play a role in the reorganization of connective tissue during inflammation. At the same time, these cells have the potential for causing disease if, for example, a phlogogenic stimulus persists, leading to a continuous and repeated influx of macrophages into tissues with the persistent secretion of enzymes. Speculation along these lines has been made by Davies *et al.*,[11] based on the example of persistent granulomatous inflammation produced by poorly digestible streptococcal cell-wall proteins.

Of great interest is the secretion of mediators of the immunologic process. It is thought that two interrelated functions of phagocytes in immunologic process are to concentrate and remove antigen and to present some molecules for recognition of T and B lymphocytes. Previous studies have analyzed this question at depth. We now find that phagocytes can also secrete molecules which have the capacity to change the response of the lymphocyte. The secretion of lymphostimulatory molecules is under

regulation in the sense that it is controlled by important extracellular factors—a particle that is phagocytosed or an activated T cell. Furthermore, the extracellular factors only appear to operate on certain maturational steps of the phagocyte. The overall evidence suggests that the secretion of lymphostimulatory molecules, and of other molecules in general, is under a rather sophisticated control which could explain the various, diverse roles of phagocytes. Our data is consistent with the hypothesis that uptake of finite amounts of antigen by phagocytes of primary and secondary lymphoid organs leads to a burst of secretion of the lymphostimulatory molecules. In the thymus the reaction could play a critical homeostatic role in regulation of T-cell maturation in accordance with the activity of the phagocytes. In lymph nodes and spleen the secretion of the lymphostimulatory molecules could play a cooperative role during induction. We also speculate that a probable mechanism of action of immunologic adjuvants is the macrophage through the release of the lymphostimulatory factor mediated as described herein.

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