

The elusive humors of the lymphocyte mediators of delayed hypersensitivity

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It is a great honor for me to be here to deliver the Robert A. Cooke Memorial Lecture. As you know, my subject this morning deals with the substances produced by lymphocytes which relate to cell-mediated immunity or hypersensitivity. Since, in this exciting area, things seem to change so frequently, perhaps I should begin with a brief discussion of what we consider to be cell-mediated immune reactions.

Such reactions include delayed hypersensitivity, immunity to certain infections, including some bacteria, viruses, fungi, and parasites, immunity to tumors in animals and probably in man, graft rejections, graft-versus-host reactions, and some aspects of the pathologic reactions seen in certain "auto-immune" diseases. Although this list encompasses responses that appear to be strikingly varied, there is a common local reaction seen in most of these: that is, an inflammatory response characterized by a mononuclear infiltration predominantly comprised of lymphocytes and cells derived from blood monocytes. In addition, we now know that the majority of the cells at this site of inflammation are nonsensitive but become secondarily involved after the initial reaction of antigen with a *few* sensitized lymphocytes. This was shown, for example, in studies by McClusky and associates¹ using cell labeling with tritiated thymidine which revealed that in the transfer of delayed sensitivity in guinea pigs most of the cells at the site of inflammation are nonsensitive, bone marrow-derived, rapidly dividing mononuclear cells of the host. Only a few are cells that were injected from the sensitive animals. Similarly, we know that a few sensitive lymphocytes will influence the behavior in vitro of a large number of nonsensitive macrophages.

By what means can a few sensitive lymphocytes, having come in contact with the sensitizing antigen, attract and involve large numbers of nonsensitive cells? An accumulating amount of evidence suggests that this comes about through the release of soluble substances with various biologic effects. Because these factors are obviously in exceedingly small supply and thus difficult to isolate and identify, the mediators, as they are called, are best described by describing their biologic activities.

It can be seen (Fig. 1) that the list of mediators is already impressively

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- 1) MIF
- 2) Chemotactic factor for macrophages
- 3) Macrophage activating factor (? same as MIF)
- 4) Macrophage aggregation factor (? same as MIF)
- 5) Chemotactic factor for neutrophils
- 6) Cytotoxic factors, i.e., lymphotoxin
- 7) Clonal and proliferation inhibition factor
- 8) Inhibitor of DNA synthesis (IDS)
- 9) Skin reactive factor (? combination of several above)
- 10) Blastogenic factor for lymphocytes
- 11) Interferon
- 12) Immunoglobulin
- 13) Transfer factor

FIG. 1. Factors produced by sensitive lymphocytes when stimulated by antigen.

long. They include a migration inhibition factor (MIF¹) which inhibits the migration of normal macrophages, a chemotactic factor, a factor which causes redness and mononuclear infiltration in the skin, factors which are cytotoxic or prevent cell growth, a blastogenic factor which causes normal lymphocytes to divide, substances with interferon-like activities, immunoglobulins and transfer factor. I should like to point out that these may indeed be effects of individual substances produced by antigen-stimulated lymphocytes, but it is also possible, in some cases, that we are observing multiple activities of a single substance.

The MIF story told from the beginning is perhaps the best way to illustrate the development of investigation into the lymphocyte mediators. In 1962, George and Vaughan² introduced the capillary tube technique to demonstrate inhibition of macrophage migration by specific antigen. With this simple and semiquantitative method, workers in several laboratories have gathered information which clearly correlated the reaction with *in vivo* delayed hypersensitivity and goes far in explaining the mechanism of the reaction. The migration of peritoneal exudate cells from previously sensitized animals is consistently inhibited in the presence of the sensitizing antigen. That this is an exquisitely specific immunologic reaction has been shown with protein antigens, with hapten-protein conjugates³ and with synthetic antigens.⁴ In early studies it was found that guinea pigs sensitized in such a manner as to produce antibody, but not delayed hypersensitivity, yield peritoneal cells which are not inhibited by the sensitizing antigen.^{2, 5}

As it was apparent that the peritoneal exudate cells used in these studies are a heterogeneous population consisting mainly of macrophages but also containing a significant number of lymphocytes, it was necessary to determine not only the sequence of events in inhibition of migration but also the contribution of the two main cell types. The first series of experiments on this question was based on our finding that, in admixtures of normal and sensitive cells in varying proportions, the sensitive peritoneal exudate cells could be diluted to a concentration as low as 2½ per cent with normal cells and still cause the entire population to be inhibited by antigen.⁶ In addition, the sensitive cells had to be viable and metabolizing to produce this effect. These results were taken as evidence that the number of specifically sensitive cells in a given area need only

be very small to involve large numbers of normal cells. To investigate whether the lymphocytes were the initial responders in the reaction with antigen, experiments were designed repeating the mixture experiments using lymph node lymphocytes as the sensitive cell population instead of peritoneal exudate cells from sensitized animals. It was found that lymph node lymphocytes from hypersensitive guinea pigs are not themselves inhibited from migrating in vitro by the sensitizing antigen, but when added to normal peritoneal macrophages they will cause the resulting cell population to be inhibited by specific antigen. The same results were obtained using spleen cells as the sensitive cell population. Bloom and Bennett,⁷ in an elegant series of experiments, showed that peritoneal exudate cells from sensitive animals freed of their lymphocyte component are no longer inhibited by antigen; when the lymphocytes were added back to the macrophages, it was again possible to observe inhibition of migration by the specific antigen. It appeared, then, that we were, in fact, observing a reaction which took place initially between the lymphocytes and antigen, and secondarily involved the macrophages; it is important to note that the macrophages are necessary for inhibition to occur as they are the cells which are inhibited.

In the light of these findings, it is interesting to make a simple but startling calculation with the figures from the first mixture experiments. If the initial sensitive peritoneal cell population contains approximately 20 per cent lymphocytes, not all of which can be specifically sensitive, and 2 per cent of these cells are sufficient to inhibit a population containing 98 per cent normal cells, it means that one sensitized lymphocyte, in the presence of specific antigen, can affect several thousand macrophages. It is easy to see, then, why the concept of a cell-to-cell reaction was abandoned and why we were stimulated to search for a soluble mediator produced by the sensitive lymphocytes. The possibility that such a mediator existed was also suggested by the results of the experiments of Johanovsky and Svejcar⁸ in which spleen explants from normal and sensitized animals were cultured in the same chamber. Such a mediator was found in 1966 by Bloom and Bennett⁷ and by me⁹ and has been called migration inhibitory factor or MIF.

It was demonstrated that lymphocytes, when stimulated in vitro by specific antigen, elaborated into the medium a soluble material which inhibited the migration of normal peritoneal exudate cells or normal macrophages. This migration inhibitory factor was produced only in the presence of specific antigen and was not found in the cell-free supernatants of lymphocytes cultured in medium without antigen or medium containing unrelated antigen. The production of the material was shown to be blocked by puromycin or mitomycin. Studies on the migration inhibitory factor have now also been carried out in several other laboratories.

MIF is first detected after 6 to 8 hours of incubation and can be found in the supernatants for as long as 4 days. It is nondialyzable, heat stable at 56 degrees for 30 minutes, and resistant to treatment with RNase and DNase. MIF elutes from Sephadex G-100 in a broad band including albumin, with the peak activity in the fractions eluting later than the albumin peak. Similar results are obtained when MIF-rich supernatants are fractionated by sucrose gradient centri-

fugation. Since MIF activity was recovered over a broad area in both methods, exact determination of molecular weight is difficult. However, both methods indicate a molecular weight in the range of 56,000 to 35,000.

To further purify MIF, disc acrylamide gel electrophoresis was chosen. When gels were polymerized in the usual manner, the fractions eluted from them were toxic to cells in culture. Methods for producing gels which yielded nontoxic fractions were devised by Dr. Heinz Remold in our laboratory. When the active Sephadex G-100 fractions which contain MIF activity were electrophoresed on acrylamide gels at pH 9.1, the MIF activity was consistently found in a fraction which contains proteins migrating in front of albumin.¹⁰

Another set of experiments was carried out by Dr. Remold in which MIF was incubated with insolubilized chymotrypsin (chymotrypsin which is covalently bound to a backbone made from a copolymer of ethylene and maleic anhydride). After the incubation, the enzyme was removed from the solution by centrifugation. Thus, the presence of the enzyme in the assay medium was avoided, and any influence of the enzyme upon the assay system was excluded. MIF was also incubated for one hour at 37° C. with neuraminidase. The neuraminidase was removed after incubation by gel filtration in Sephadex G-100. The results of these experiments showed that chymotrypsin and neuraminidase completely abolish MIF activity.

Further information about this mediator was obtained by cesium chloride density gradient centrifugation. MIF was put in a CsCl solution and spun in an ultracentrifuge at 45,000 r.p.m. for 36 hours until a density gradient of CsCl had been established. Substances like proteins sediment in a zone of the same density. It is therefore possible to separate substances with different buoyant densities from each other in such a gradient. These experiments showed that MIF has a greater buoyant density than pure protein, which is consistent with its being a glycoprotein.

The results of Sephadex fractionation, acrylamide gel electrophoresis, enzyme degradation, and CsCl gradient centrifugation strongly suggest that MIF is a glycoprotein of molecular weight 55,000 to 35,000.

It might be said that the effect of a few sensitive lymphocytes producing a soluble material which then involves large numbers of nonsensitive macrophages serves as a biological amplifier. An *in vivo* reaction of this type between sensitive cells and antigen resulting in a large cellular reaction could produce either benefit for the host, if the macrophages are activated and ingest and destroy pathogenic organisms, or it could end in tissue damage. The possible role of MIF in *in vivo* delayed reactions and the effect of lymphocyte-produced substances on the biology of macrophages are questions of great interest which are open to be explored.

Bennett and Bloom¹¹ began such investigation with experiments in which they injected MIF-rich fractions into the skin of normal guinea pigs and observed erythematous and indurated reactions which were apparent at 3 to 5 hours and maximal at 8 to 12 hours. When examined histologically, the reactions contained an accumulation of mononuclear cells and were similar in appearance to delayed hypersensitivity skin reactions. Pick and associates¹²

have begun analysis of the skin-reactive material and have reported that the active substance elutes from Sephadex in the same area as MIF activity and that inhibitors of protein synthesis suppress its production. At this juncture, the material is called skin-reactive factor, but it is likely that the skin reaction is the result of the action of several mediators, including chemotactic factor and MIF.

Since the early stages of the MIF work, we have all been concerned with the relationship of the *in vitro* studies to *in vivo* reactions. One promising possibility was that the MIF-active supernatants might be chemotactic for mononuclear cells. To this end we entered into an active collaboration with Dr. Peter Ward, and throughout the MIF studies, samples from experiments were tested by him for chemotactic activity. The chemotactic assay used is the Boyden chamber or micropore filter method in which cells migrate through the filter toward the opposite compartment containing the chemotactic material. Chemotactic activity is measured by counting the number of cells that have migrated completely through the filter in five fields examined with light microscopy.

With this technique, it was quickly apparent that culture fluids from sensitized lymphocytes that had been incubated in the presence of antigen were chemotactic for mononuclear cells. The activity was dose dependent on the amount of culture fluid tested.¹³

To assess the requirement for antigen in the appearance of chemotactic activity in culture fluids, o-chlorobenzylchloride (OCB-BCG) was added in two experiments to supernatants from sensitized cells after the incubation period had terminated and the cells had been removed. The mere presence of antigen in these supernatants was not associated with chemotactic activity—antigen had to be present during the period of incubation with lymphocytes to stimulate the production of chemotactically active material.

The production of chemotactic activity was found to be immunologically specific, and activity was still present after heating at 56° for 30 minutes.

Next, we found that MIF and chemotactic factor both elute from Sephadex G-100 in a broad area with the highest activity in the fractions eluting immediately after the albumin peak. These findings were substantiated in experiments with the use of sucrose density gradients. Both methods indicate a molecular weight in the range of 35,000 to 55,000 for both factors.

Chemotactic activity for mononuclear cells is consistently eluted from acrylamide gel fractions containing albumin and is not found in the prealbumin region. In contrast, MIF activity is regularly recovered from the prealbumin fraction. Occasionally, MIF is found in both the albumin and the prealbumin regions. In several experiments it was clearly demonstrated that prealbumin fractions with a high level of MIF activity had no chemotactic activity and, vice versa, that a fraction containing albumin with no MIF activity had good chemotactic activity. It should be emphasized that throughout these experiments, both biological activities were assayed with the use of aliquots of the same sample.¹⁴

When the fraction containing chemotactic activity was placed with the cells in the upper chamber, it inhibited, as expected, the chemotactic effect of

the chemotactic fraction in the lower chamber by preventing the formation of a chemotactic gradient. In contrast, the presence of MIF in the upper chamber did not prevent the effect of chemotactic fraction in the lower chamber. These and other experiments strongly support the existence of two separate substances—one, a chemotactic factor, the other, MIF.

These results made it quite clear to us that antigen-stimulated lymphocytes produce a complex mixture of mediators, some of which appear to be distinct molecules. It will be of great interest to determine how many of the other lymphocyte factors, such as cytotoxic factor, skin-reactive factor, cloning inhibition factors, blastogenic factors, interferon activity, etc., can be distinguished by similar means.

The next group of lymphocyte factors to be considered can be called cytotoxic factors. Some time ago it was established by Govaerts and by Rosenau and Moon that in the transplantation system lymphocytes from a tissue-sensitized animal are cytotoxic in culture to cells which bear the same antigens as the sensitizing tissue. In these studies and in others which confirmed them, it seemed that direct contact between the sensitized lymphocytes and the antigen-bearing target cells was required for toxicity or cell killing to occur. More recently, Ruddle and Waksman,¹⁵ and subsequently others, have published studies which indicate that, at least under some experimental conditions, soluble materials produced by sensitized lymphocytes are toxic to culture or target cells. Ruddle and Waksman began by showing that lymph node cells from rats sensitized with soluble antigens, when placed in culture together with the sensitizing antigen and rat embryo fibroblasts, would kill the fibroblasts. This established that lymphoid cells from animals with delayed hypersensitivity reacted in culture with antigen and, as a result of that interaction, killed other cells in the same culture. Using hapten-protein conjugates, the same workers showed that the cytotoxic effect was subject to the same carrier-type specificity as other cell-mediated immune reactions but that it did not correlate with the Arthus reaction. They went on to show that when the target cells were preincubated with antigen, washed, and then added to sensitive lymph node cells in culture, the target cells were not killed, indicating the lymphocytes produced toxicity as a result of their interaction with antigen. Furthermore, in experiments in which sensitive and normal lymphocytes were mixed in varying proportions, cytotoxicity was directly proportional to the number of sensitized cells, suggesting that the sensitized cells did not confer cytotoxic properties to normal lymphocytes.

Next, Ruddle and Waksman gave their evidence for mediation of the cytotoxic reaction by a soluble lymphocyte mediator in experiments in which supernatants from cultures of sensitive lymphocytes with antigen, in this case purified protein derivative (PPD), were used as culture medium for rat fibroblast monolayers. Cytotoxicity was observed in cultures containing supernatants from sensitive lymphocytes with antigen, but it was not observed in those containing supernatants from normal cells with antigen or those from sensitive cells with no antigen. Thus, they¹⁵ demonstrated that the specific interaction of sensitive lymph node cells, presumably lymphocytes, and antigen resulted in

the production and release of a cytotoxic material which damaged target cells by a nonimmunologic mechanism, possibly by activating the lysozomal enzymes in the target cells.

Perhaps the most extensively investigated group of cytotoxic factors are the lymphotoxins described by Granger.¹⁶ These are, in the main, substances obtained following exposure of lymphocytes to mitogens, such as phytohemagglutinin (PHA), which stimulate the cells nonimmunologically. Granger has reported that supernatants from such lymphocyte-PHA cultures are highly toxic for mouse L cells, a long-term cell line. He has successfully used lymphocytes from various animal species and from human beings to produce the lymphotoxins.

Human lymphotoxin is produced under stimulation by PHA if the lymphocytes are actively metabolizing at 37° C. It can be detected as early as 3 to 6 hours, and its production is inhibited in the presence of puromycin or hydrocortisone. In addition, Kolb and Granger¹⁷ have reported that stimulated human lymphocytes will produce lymphotoxin following x-ray treatment, suggesting that the cells do not have to be dividing to produce the cytotoxic material.

Lymphocytes stimulated by antigen have also been shown to produce soluble substances which prevent the growth of cells in culture without necessarily killing them. The relationship of these to the cytotoxic materials is being investigated.¹⁸

Another biologically active substance produced by lymphocytes is a blastogenic factor which has been described by Valentine and Lawrence.¹⁹ In their experiments, human lymphocytes from persons sensitive to tuberculin were incubated with PPD and the supernatants from the cultures harvested. These supernatants were added as media to cultures of lymphocytes from normal tuberculin-negative individuals, and the ability of the normal lymphocytes to divide or take up ³H-thymidine was assessed. As one would expect, the normal cells did not transform or take up thymidine when PPD was added to them. However, supernatants from PPD-stimulated sensitive cells caused these normal lymphocytes to divide and take up thymidine. This material, the blastogenic factor, is different from the other mediators we have been discussing in that it appears to be antigen dependent. That is, not only does one need antigen present to stimulate the release or production of the material by sensitive cells, but antigen must also be present for the factor to act on the nonsensitive cells. This suggests that the blastogenic factor may be an antigen-antibody complex, but no information is available at present on this point. This blastogenic factor is nondialyzable, and its activity is destroyed by heating at 56° for 30 minutes. Further, it is nonsedimentable at 100,000 × g. It should be pointed out that other laboratories have described factors from stimulated lymphocytes which have blastogenic activity, and some of these factors have been reported not to need antigen for their action on normal cells.²⁰

Another product of the sensitized lymphocyte is interferon. Several investigators have described the production of material with the characteristics of interferon by lymphocytes following stimulation with mitogen such as phytohemagglutinin. Green, Copperband, and Kibrick²¹ have reported that human

lymphocytes, when they are taken from sensitive individuals, produce interferon following stimulation with an antigen to which the donor is sensitive. This interferon was produced after 4 to 7 days of culture, whereas interferon produced with mitogen stimulation was usually observed earlier. There appears to be a dissociation between transformation of lymphocytes and the production of interferon since as much interferon was made by either PHA-or PPD stimulated lymphocytes, while much more transformation is seen with PHA than with antigen. As with other interferons, the material produced by human cells is species specific and will act to prevent viral replication only when human cells are used in culture; it does nothing when added to mouse cells. This is quite different from lymphotoxin, for example, which is not species specific. The interferon produced by lymphocytes under PPD stimulation is nondialyzable, stable at wide pH range, nonsedimentable at $100,000 \times G$, and its molecular weight has been estimated at 30,000. It is resistant to treatment with RNase and DNase and is destroyed by trypsin; this is consistent with other interferons. Whether or not the interferon produced by lymphocytes is active in resistance to viral infection in vivo is not known at present, but clearly it is a good possibility that such would be the case. We await the results of the studies to determine this point with great interest.

Another factor that is obtained from sensitized lymphocytes is the "transfer factor" of H. S. Lawrence.²² Although it can be released from sensitive cells when they are incubated for one hour with antigen, it is important to note that the transfer factor differs from the lymphocyte-produced mediators discussed today in that it is usually extracted from sensitive cells without the stimulation of antigen. Also, it is a dialyzable substance. The properties of this most interesting material have recently been reviewed in detail by Dr. Lawrence in *Advances in Immunology*.

The last of the substances produced by lymphocytes during stimulation by antigen is the one best known to all—antibody. We have always assumed that the cell populations usually used for the production of mediators, especially those taken from lymph nodes, contain cells that produce humoral antibodies. However, cells in suspensions cultured in vitro for a short time do not produce amounts of antibody that are readily detectable by conventional means. We were especially interested to determine whether, under the conditions we use to produce MIF and other mediators, antibody was also being made. Dr. O. L. Meyers, in our laboratory, studied the production of anti-bovine gamma globulin (BGG) antibodies in lymphocyte cultures under the same conditions routinely used to produce MIF. The presence of newly formed anti-BGG antibody was measured in these studies by a coprecipitation technique. In this method, cells are incubated in the presence of ^3H -leucine, and any newly formed antibody is thereby rendered radioactive. Known amounts of nonradioactive BGG-anti-BGG complex is then added to coprecipitate any labeled antibody, which, if present, can then be measured. Such cultures are first treated with antigen-antibody complexes made with a different antigen to remove any proteins which might nonspecifically bind to such complexes. Using this method, Meyers showed that lymphocytes cultured with specific antigen (BGG) for 24 hours under the

conditions used to produce MIF also produced antibody to BGG. It was interesting that lymphocytes sensitive to BGG but not stimulated in vitro with BGG also made a small amount of antibody but less than antigen-stimulated cultures. These findings bring out two important points. The first is that antibody is also present when lymphocytes are being cultured with antigen to produce mediators. The second is that when supernatants from lymphocyte cultures are used without prior fractionation, any effect observed may be complicated by the presence of antigen-antibody complexes.

We have covered rather quickly some of the recent work which reflects the present state of our knowledge of lymphocyte mediators. Much more work is ahead of us in sorting out these different materials and in determining the significance of each in in vivo phenomena. The field is bound to become more complicated, so much so that one might invoke the old saw, "It will get worse before it gets better"; but it is also bound to get better, and, as I believe you would agree, the promise in that getting better makes the work worthwhile.

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