MACROPHAGE-DIGESTED ANTIGEN AS INDUCER OF DELAYED HYPERSENSITIVITY*

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While most antigens capable of inducing antibody responses can, under appropriate conditions, also induce delayed reactivity, some substances are preeminently invokers of delayed hypersensitivity while they induce antibody synthesis poorly or not at all. Generally these are small molecular entities, or larger molecules that may be only partially recognized as "foreign" by the responder. Examples of the first are arsenobenzoate-*N*-acetyl tyrosine (1) and small synthetic peptide copolymers of tyrosine and glutamic acid (2). In both of these cases, expanding their molecular size and complexity decreases their efficacy as inducers of delayed reactivity, while improving their capacities to induce antibodies. Examples of the second kind are gelatin (3), slightly altered homologous serum proteins (4), and a peptide derived from adrenocorticotropin (ACTH) (5), all of which induce good delayed reactivity and poor antibody synthesis. Delayed contact hypersensitivity resulting from the spontaneous conjugation of applied chemicals (haptens) with autologous epidermal protein carriers (6, 7) illustrates this point also; antibody production is a minor response in these cases.

Complete Freund adjuvant, a water-in-oil emulsion containing mycobacterial bodies, stimulates the development of delayed reactivity to antigens incorporated in it. This activity of mycobacteria in favoring a delayed cellular response has been related to a glycolipopeptide of the cell wall (8, 9). Mycobacteria or their glycolipopeptides elicit the appearance of epithelioid cells, which presumably arise from macrophages. In the process of transforming to the epithelioid cell, the macrophage undergoes "activation" characterized by an increase in numbers of lysosomes and a greater propensity for phagocytosis (10, 11). Such activated macrophages may have a role in processing, i.e. digesting, macromolecular antigens to fragments which, like the small molecular compounds mentioned, are particularly apt in inducing delayed reactivity, but are poor as stimuli for the antibody response.

The thought that smaller structures possibly containing only single immunogenic determinants may be better inducers of delayed reactivity than are multideterminant

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molecules coincides with a current concept that the antibody response may require two responding units. One is a thymus-derived lymphocyte which recognizes and binds some determinants, and holds the molecule for interaction of another of its determinants with a second (bone marrow-derived) cell. The latter is able to respond to the second determinant with the production of antibody (12–14).

The thymus-derived cell may be the effector cell for delayed hypersensitivity (15). If it binds the determinant of a molecule which contains no other immunogenic unit, then the response may be limited to that of which this cell is capable, i.e., delayed hypersensitivity.

The circumstances that could lead to this restricted kind of response might be, as suggested above, the primary use of a small molecular immunogen as a sensitizing agent, or sensitization with a larger molecule of which only restricted portions are immunogenic, or the conversion of a multideterminant antigen to simpler fragments through the increased digestive activities of activated macrophages.

In order to test this last thesis, we used a system in which normal and activated macrophages were allowed to phagocytose and digest sheep red blood cells for various periods of time. The contents of the disrupted macrophages were tested for their abilities to induce antibodies and delayed hypersensitivity. After longer intervals of digestion, the stimulus for the former disappeared, while that for the latter persisted.

Materials and Methods

Harvesting of Macrophages.—Peritoneal macrophages were obtained from randomly bred albino guinea pigs of either sex weighing 350–500 g. The animals were sacrificed, the abdominal skin was reflected, and 20 ml of Hanks' basic salt solution (HBSS)[1] containing 10 units/ml of preservative-free heparin (Calbiochem, Los Angeles, Calif.) was pipetted into the peritoneal cavity via a slit in the abdominal wall. The abdomen was gently massaged and the peritoneal fluid was withdrawn with a pipette. Normal macrophages were collected from guinea pigs which had received no peritoneal stimulation. Activated macrophages were collected from the cavities or omenta of animals which had received 1.0 ml of complete Freund adjuvant (CFA) (Difco Laboratories, Inc., Detroit, Mich., H37Ra, 1.0 mg/ml) in physiological saline (1:1) intraperitoneally 3 or 5 days previously.

Peritoneal cells were spun down at 500 rpm for 5 min in Falcon plastic tissue culture tubes (Falcon Plastics, Los Angeles, Calif.). The cells were washed with 5 ml of HBSS containing 1% normal guinea pig serum (GPS), 50 μg/ml sodium penicillin, 50 μg/ml streptomycin, and 5 μg/ml neomycin (PSN). If red cells were present they were lysed by a second washing in one part HBSS to two parts distilled water. The cells were then suspended to 3×10^6 cells/ml in medium 199 in Hanks' solution with 15% GPS and PSN.[2]

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[1] *Abbreviations used in this paper:* BCG, bacille Calmette Guerin; CFA, complete Freund adjuvant; GPS, normal guinea pig serum. HBSS, Hanks' balanced salt solution; IFA, incomplete Freund adjuvant; PSN, 50 μg/ml sodium penicillin, 50 μg/ml streptomycin, and 50 μg/ml neomycin; SRBC, sheep red blood cells.

[2] All media were purchased from Grand Island Biological Company, Grand Island, N.Y. Normal guinea pig serum was obtained from Hyland Laboratories, Burbank, Calif.

Macrophages were collected from the omentum by separating the membrane from adherent fat and chopping it finely. The fragments were gently pipetted several times in HBSS with 1% GPS to release the cells. Tissue fragments were allowed to settle for 5 min and the supernatant was decanted for treatment as described for free peritoneal cells.

Preparation of Sheep Erythrocytes.—Sterile sheep red blood cells (SRBC) in Alsever's solution were washed three times in sterile physiological saline. SRBC to be used for skin testing were suspended at final concentrations of 2 or 5% v/v in physiological saline. The SRBC to be added to peritoneal cell cultures, after the three saline washings, were washed once with HBSS and suspended to a final concentration of 2% v/v in medium 199 with 15% GPS and PSN. Fresh preparations of SRBC were made for each experiment.

Incubation of Macrophages with Sheep Red Cells.—For macrophage digestion experiments, 2 ml of fresh cell suspension (6×10^6 cells) in a tissue culture tube were mixed with 1 ml of 2% SRBC (2×10^8 cells). The tubes were gassed with a 5% CO_2:95% air mixture and incubated at 37°C on a drum roller (1 rev/10 min) for periods of 1 and 7 hr, and in some experiments for 12 and 24 hr. The tubes were then spun at 800 rpm for 10 min, and the culture medium was removed. The unphagocytosed SRBC were lysed by a brief washing in HBSS in distilled water 1:2. The macrophages were then resuspended in 1.3 ml of HBSS containing antibiotics and were frozen and thawed three times.

For studies of the uptake of SRBC by macrophages, 1.5 ml of cell suspension (4.5×10^6 cells) was placed in Leighton tubes or small Falcon Petri dishes, 0.75 ml of 2% SRBC (1.5×10^8 cells) was added, and the mixtures were incubated in 5% CO_2–air for various periods. SRBC and all other nonadherent cells were then washed away with saline, and the macrophages were dried and fixed for 3 min in anhydrous methanol. The cells were stained with Wright-Giemsa and examined for ingested SRBC.

Sensitization of Guinea Pigs.—Albino guinea pigs were injected with test substances in 0.4 ml total volume, divided between the two hind footpads. The materials used were: (*a*) disrupted macrophage–SRBC preparations mixed with water–oil emulsion; (*b*) control injections of antigen consisting of 2% SRBC (4×10^7 cells) in incomplete or complete Freund adjuvant; (*c*) numbers of SRBC estimated to be phagocytosed in the experimental mixtures, added to heat-inactivated macrophages or injected alone, as will be described under Results.

Antibody Titrations.—2 ml of blood were collected from guinea pigs before skin testing, at 3 wk after sensitization. Microhemagglutination tests were performed by 2-fold serial dilutions.

Skin Tests.—After bleeding, guinea pigs were injected intradermally in the flank with 0.1 ml of 5% SRBC in saline; this concentration of cells caused little or no nonspecific irritation. The test sites were read for diameter of thickening and erythema at 4, 24, and 48 hr. Some sites were removed at the 24 hr reading, fixed in 10% formalin, sectioned, and stained with hematoxylin and eosin for histologic study.

RESULTS

Responses to SRBC in Incomplete and Complete Freund Adjuvant.—Patterns of antibody and skin responses to SRBC were established in groups of seven animals injected with 0.4 ml of 2% cells in each of the adjuvants. At 21 days, all animals in both groups had hemagglutinating antibodies (Table I, Fig. 1). Undiluted normal sera were negative.

Skin tests (Fig. 1) with 5% SRBC in saline elicited reactions in the incomplete Freund group which were at their peak at 4 hr, had decreased by 24 hr, and dropped further, in some cases disappearing, by 48 hr. The complete Freund group responded with larger indurated reactions which for the most part showed central necrosis. These reactions increased in diameter between 4 and

24 hr, and dropped off to some extent at 48 hr. These results are of the kind previously reported by Boyden (16) and follow the general pattern seen with soluble antigens as well (e.g., 17).

Responses to SRBC Ingested by Macrophages.—The cellular compositions of peritoneal exudates are shown in Table II. Exudates from guinea pigs which had received complete Freund adjuvant intraperitoneally 3 or 5 days earlier contained many more cells than did those from normal animals. The majority

TABLE I

Antibody Incidence and Mean Titers in Guinea Pigs Receiving 2% SRBC in Incomplete or Complete Freund Adjuvant

Freund adjuvant	No. of guinea pigs tested	Per cent of positive hemagglutinins	Range of titers	Mean titer
Incomplete	7	100	1:4–1:256	76.7
Complete	7	100	1:16–1:256	96.0

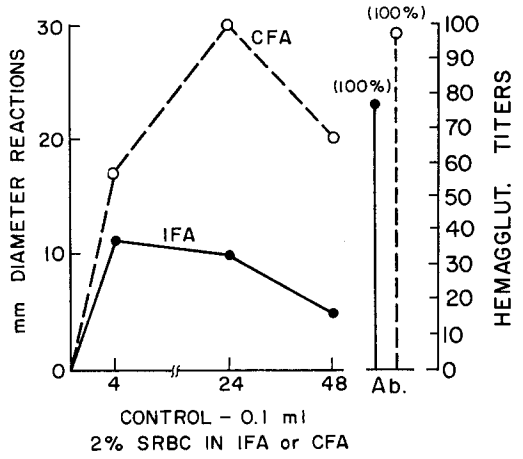

FIG. 1. Skin reactions and antibody responses of guinea pigs to sheep erythrocytes in complete (CFA) and incomplete (IFA) Freund adjuvant.

of the granulocytes were eosinophiles; these were fragile in culture and were never seen to contain phagocytosed SRBC.

Normal peritoneal cells were incubated with SRBC for 24 hr, and samples were prepared for injection into normal guinea pigs after 1, 7, and 24 hr. Each animal received the material from 10^6 disrupted macrophages in a volume of 0.4 ml of water–oil emulsion. The recipients were examined for antibodies and delayed reactivity to SRBC 21 days later. Results are shown in Table III and Fig. 2.

The 1 hr samples induced antibodies in 5 of 10 animals, with titers ranging from 1:32 to 1:128 (mean, 24.6).

After 7 hr of incubation, 2 of 10 animals responded with sparse antibodies, and at 24 hr the preparation failed to induce antibodies in 3 animals injected.

The skin reactivities developed by these groups are shown in Fig. 2. The group receiving the 1 hr sample showed a pattern suggesting Arthus reactivity, perhaps combined with delayed. The 7- and 24-hr samples induced reactions of more evident delayed type that increased in size between 4 and 24 hr. Thus, as immunogenicity in respect to antibody induction declined with longer periods

TABLE II

Cellular Composition of Normal and Adjuvant-Induced Peritoneal Exudates

Guinea pig cell donor	Average No. of cells/ guinea pig	Per cent of cell composition		
		Macrophages	Lymphocytes	Granulocytes
Normal	16×10^6	80–90 (occ. 65)	5–15	2–5 (occ. 30)
3 days post CFA i.p.	65×10^6	55–75	5–10	20–35
5 days post CFA i.p.	89×10^6	50–70	13–15	15–35

TABLE III

Antibody Incidence and Titers in Guinea Pigs Receiving Disrupted Normal Peritoneal Macrophages Incubated with Sheep Erythrocytes

Incubation period	No. of guinea pigs tested	Per cent responding with hemagglutinins	Range of titers	Mean titers
(*hr*)				
1	10	50	32–128	24.6
7	10	20	4–8	2.2
24	3	0	0	0

of intramacrophagic residence of the SRBC, the ability to engender delayed reactivity appeared to increase.

Cells from guinea pigs treated with adjuvant 3 or 5 days earlier were tested after 1, 7, or 12 hr of incubation with SRBC. Again, tests for antibodies and skin reactivity were made at 21 days. There were no significant differences seen in the tests with cells collected at 3 or 5 days after adjuvant, whether from peritoneal exudates or omenta. These results have been combined for discussion (Fig. 3, Table IV).

The 1 hr samples induced antibodies in 65% of 21 animals, with titers of 1:2–1:32 (mean, 13.1). With the 7 hr mixture 56% of 26 animals showed titers in this same range (mean, 11.7), but by 12 hr none of 5 injected guinea pigs produced detectable antibody.

The skin reactions induced by these three incubation mixtures are shown in Fig. 3. The 1 hr group showed reactions of Arthus type, while the animals which had received the 7- and 12-hr samples revealed delayed-type reactivity.

Histologic studies of skin test sites were made of members of groups that had

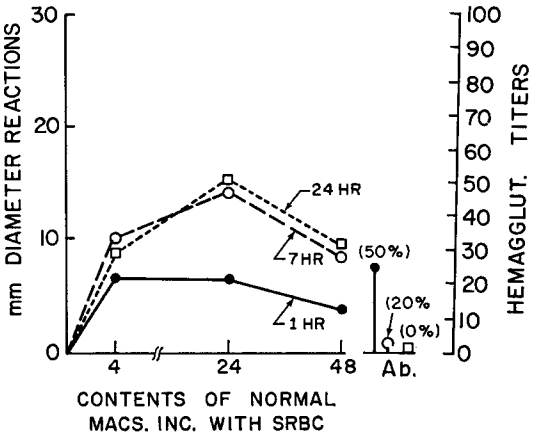

FIG. 2. Skin reactions and antibody responses of guinea pigs to sheep erythrocytes digested by normal macrophages for periods of 1, 7, and 24 hr.

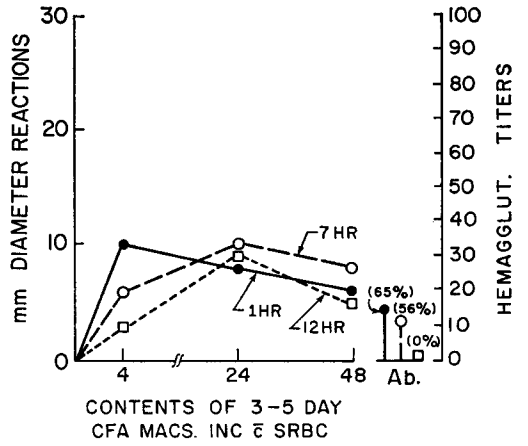

FIG. 3. Skin reactions and antibody responses of guinea pigs to sheep erythrocytes digested by adjuvant-activated macrophages for periods of 1, 7, and 12 hr.

been sensitized with the 1 and 7-hr incubation mixtures of normal macrophages and SRBC. The sites were removed 24 hr after skin test injection, and stained with hematoxylin–eosin. The 1 hr sites showed light mononuclear infiltration of the dermis and a small degree of perivascular cuffing in 1 of 5 cases; many of these cells were polymorphonuclears. In the 7 hr group, the dermal mononuclear

infiltrate was moderately heavy, as was perivascular cuffing, in which a small percentage of cells were polymorphonuclears. 24-hr SRBC skin test sites from normal control guinea pigs showed very light deposition of mononuclear and polymorphonuclear cells. These results indicate the occurrence of delayed reactions in the 7 hr group, and suggest mixed Arthus and delayed reactivity in the 1 hr animals.

These observations do not distinguish the influence of adjuvant-treated from normal macrophages in altering the immunogenic capacities of sheep erythrocytes in the direction of inducing delayed hypersensitive reactivity. However, they do indicate that continuing intramacrophagic residence of SRBC results in a loss of capacity to induce antibodies and in a corresponding propensity to evoke delayed hypersensitivity as the major response.

Among the questions that arose in the course of this work, one concerned the dose of erythrocyte material contained in disrupted macrophage preparations.

TABLE IV

Antibody Incidence and Mean Titers in Guinea Pigs Receiving Disrupted Adjuvant-Exposed Peritoneal Macrophages Incubated with Sheep Erythrocytes

Incubation period	No. of guinea pigs tested	Per cent responding with hemagglutinins	Range of titers	Mean titers
(*hr*)				
1	21	65	2–32	13.1
7	26	56	4–32	11.7
12	5	0	0	0

A small number of red cells might itself dispose the recipient to develop discernible delayed reactivity while not giving rise to antibodies. We also wished to know whether an appropriately small dose of erythrocytes, equal to that phagocytosed, when incubated with killed macrophages might have the immunogenic properties described; i.e., whether the disrupted cells might exert an adjuvant effect (18). For these purposes, efforts were made to estimate the numbers of erythrocytes ingested by normal and adjuvant-activated peritoneal exudate cells under the conditions outlined.

Phagocytosis of SRBC by Macrophages In Vitro.—1 ml suspensions of peritoneal exudate cells (normal or adjuvant-activated) in Leighton tubes were incubated with 0.5 ml of a 2% suspension of SRBC, and at various times cover slips were removed, washed, and stained. For each sampling at least 500 macrophages were scored for the number of cells containing SRBC, and the number of these per cell (Fig. 4). Phagocytosis by normal macrophages reached 20% at 1 hr and this figure remained constant at 7 hr, falling off to 10% by 12 hr. The average number ingested was 1.5 per phagocytosing cell.

Cells of animals treated with complete Freund adjuvant ingested SRBC more

vigorously. 15% of those harvested 3 days after adjuvant treatment contained SRBC after 1 hr of incubation, but this number increased to 44% at 7 hr, and dropped to 15% by 12 hr. In cultures of cells from animals injected 5 days previously with complete Freund adjuvant, 23% of the macrophages contained SRBC at 1 hr and 70% at 7 hr. In both groups the number of SRBC per individual macrophage was increased over that seen in the normal; some cells contained as many as 15–20 discernible erythrocytes, with an average of about 3 per phagocytosing macrophage.

These figures permit only crude estimates of the numbers of erythrocytes that may have been ingested and digested during the total periods before the

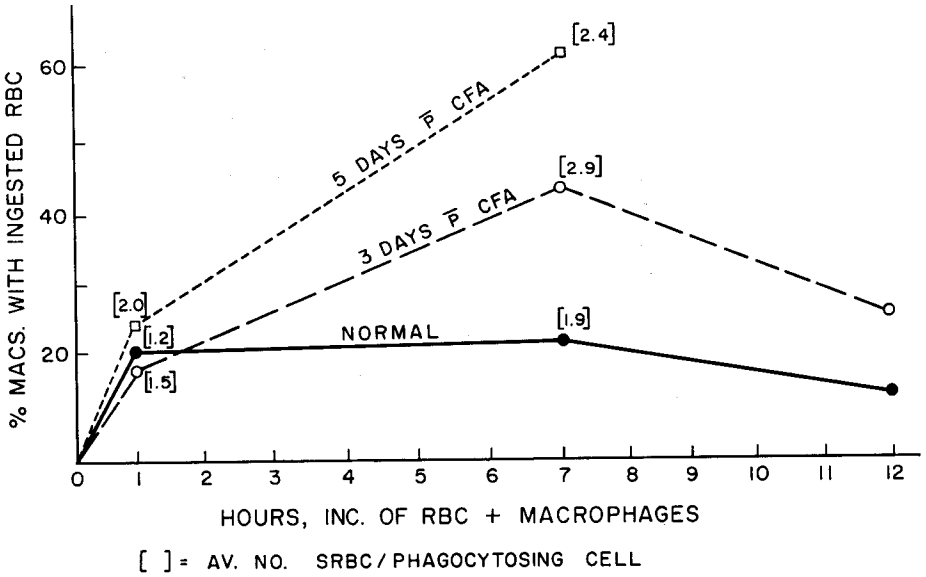

FIG. 4. Phagocytosis of sheep erythrocytes by peritoneal macrophages from normal and adjuvant-treated guinea pigs.

macrophage samples were disrupted for injection into animals. More precise estimates will require the use of labeled red cells, with frequent assays of macrophage contents and non-erythrocyte–bound label in supernatants. For present purposes, however, the figure of one SRBC per three macrophages was used. On this basis, 3×10^5 SRBC per guinea pig was used as a test dose; this is twice the calculated number of phagocytosed erythrocytes administered to each animal in the experimental groups. This dose of cells in incomplete Freund adjuvant was tested in five guinea pigs; at 21 days none gave evidence of either antibody or skin responses.

The same numbers of SRBC were mixed with heat-killed normal or adjuvant-activated peritoneal cells in concentrations equal to those used in the experi-

ments described. The cell suspensions were heated at 80°C for 30 min, and were judged to be inactive by their failure to take up erythrosin B. The killed macrophage suspensions from normal animals, or from animals pretreated 3 days earlier with adjuvant, were mixed with SRBC and incubated for 1 hr. These preparations were not washed free of SRBC, but were otherwise treated and injected as described for living cell preparations. None of 11 guinea pigs injected with these mixtures revealed antibodies or skin reactivity at 21 days.

Similar preparations were incubated for 7 hr. Of 12 guinea pigs receiving these mixtures, 1 animal showed agglutinins to a titer of 1:8, and none developed skin reactivity.

DISCUSSION

The rationale for these experiments arose from the question of how mycobacterial adjuvant may influence the induction of delayed reactivity to antigens incorporated in it. There is considerable evidence from diverse sources that small molecular immunogens used as primary sensitizing agents are especially apt in evoking this reactivity (1, 2) as are homologous proteins (3–5), of which the immunogenic entities may be minor constituents of the molecule.

There is evidence also that macrophages may eventually destroy the antigenicity of ingested substances insofar as their ability to induce antibody formation is concerned (19–23), although this is not necessarily so. These cells may degrade most of the antigen which they ingest, but in some cases they retain a small fraction of it for some time, and this is suitable for induction of antibodies (24–26). In fact, this retained fraction may be particularly evocative of antibodies.

There are indications that cells from animals treated with mycobacteria are more actively phagocytic than are normal macrophages (10) and also more active enzymatically (11). Such cells (e.g., bacille Calmette Guerin (BCG)-induced alveolar cells from rabbits) were found to be more destructive of ingested bacterial antigens than were oil-induced peritoneal cells when tested, after 5 hr of incubation, for antibody-inducing properties in mice (19). The former cells contained more β-glucuronidase, acid phosphatase, cathepsin, acid ribonuclease, and lysozyme than the latter (27).

Thus, there is information available to support one aspect of the thesis that antigenic substances may be degraded by macrophages beyond the point of usefulness as evokers of antibodies, and that this may proceed more briskly in cells obtained from animals that have been exposed to mycobacteria. The next question was whether such degraded antigens may have retained their capacity to induce delayed hypersensitivity. The results described indicate that with sheep erythrocytes this is the case. When red cells have been incubated for 7–24 hr with macrophages in vitro, the disrupted phagocytes yield products which are poor immunogens for antibody induction, but which can evoke delayed reactivity.

We have not so far found this effect on immunogens to be a more rapidly occurring one in cells activated by pretreatment of animals with mycobacterial adjuvant. A possible reason for this is in the time allowed for activation of macrophages; 3 days after treatment of the donor with adjuvant may be too late, in view of the fact that animals primarily injected with antigen in adjuvant already show delayed skin reactivity by the 3rd or 4th day (28). Perhaps more important, the intervals for sampling macrophage–antigen preparations after mixing must be more frequent; differences in the effects of normal and activated cells may well become evident between the 1- and 7-hr periods used in this work. These points, and others concerned with the nature of the antigenic fragments recovered from digesting cells, are being pursued.

A theoretical basis for these observations can be related to current proposals (12–14) that antibody induction by some antigens requires the interaction of a recirculating thymus-derived lymphocyte with a bone marrow-derived cell which is the precursor of the antibody synthesizer. The first may recognize and bind a determinant in a molecule, anchoring it so that the second cell can interact with another determinant to which it eventually responds with antibody. If the antigen presented to the responding animal is a small molecule containing perhaps a single determinant (e.g., arsenobenzoate-*N*-acetyl tyrosine), or is a macromolecular antigen that is quickly phagocytosed and fragmented by the hydrolases of macrophages that have been activated as by mycobacterial adjuvant, then the thymus-derived recognition cells would be concerned in the response, while the potential antibody-synthesizing entities would not become involved. Obviously, there would usually be intermediate situations in which both responses could occur.

One may question why, in the animal, the use of mycobacterial adjuvant often results in good antibody synthesis as well as delayed reactivity. The answer may lie in the distribution of cellular areas influenced by the lipoidal mycobacterial-activating substance. Water-soluble antigens may quickly disseminate to lymphoid tissues beyond the reach of this substance to induce antibody production.

SUMMARY

Sheep erythrocytes ingested by guinea pig peritoneal macrophages in vitro, and permitted to undergo digestion for various periods, were found after some hours to lose the capacity to induce antibodies while gaining the ability to invoke delayed hypersensitivity. These observations may be related to the known predilection of small molecular immunogens to act as good inducers of delayed reactivity and poor stimulators of antibody. They may be related also to the activity of mycobacterial adjuvant as a vehicle for the induction of delayed hypersensitivity on the basis that this melange activates macrophages to phagocytose and enzymatically degrade macromolecular antigens rapidly. The thesis that small fragments of antigenic molecules may preferentially invoke

hypersensitivity can be interpreted on the basis of current concepts of multicellular involvements in immune responses.

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