THE ROLE OF MACROPHAGES IN ANTIBODY FORMATION IN DRUG-SUPPRESSED MICE

by

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INTRODUCTION

The initial plan of this project was to employ immunosuppressive drugs as tools for examining the role of the various cell types involved in antibody formation. Particular attention was to be focused on the inductive phase of the immune response. The work of Gallily and Feldman appeared to be a good model since they utilized a cell transfer system in which the recipients were immunosuppressed by X-irradiation. Macrophages from normal mice which had engulfed Shigella antigen *in vitro* were capable of eliciting a specific agglutinin response in X-irradiated recipients (1). A similar transfer technique has been employed in this project for the purpose of examining the role of the macrophage in triggering antibody formation in animals treated with immunosuppressive drugs.

As early as 1897, Metchnikoff observed that macrophages take up and store foreign materials, and advanced the concept that they must be involved in cellular immunity (2). Whether or not this involvement is essential for the development of humoral immunity has not been resolved.

The earliest concrete evidence that macrophages were active and necessary in the induction of the immune response came from Fishman's demonstration that RNA from macrophages, previously incubated with bacteriophage, induced lymphoid cells to produce anti-phage antibody (3,4,5,6). The identification of this RNA as messenger was questioned when antigen components were detected in the inducing preparations (7,8). Garvey and Campbell reported that labeled residual antigen isolated from rabbit livers was complexed to RNA (9). Antigen

complexed with RNA appeared to be more immunogenic than the original proteins (10,11). The RNA portion was essential since ribonuclease destroyed the immunogenicity of the complex (4,7).

Fishman's basic findings concerning inducer have been confirmed, (10,11) but the nature of the triggering material derived from macrophages has remained an open question. In an attempt to determine whether this RNA was messenger, Adler et al. (12) extracted RNA from the peritoneal exudate of rabbits of one allotype and incubated it with lymph node cells from another. Since the antibody produced was of donor allotype, they concluded that the RNA was responsible for transmitting the information for synthesis of the immunoglobulin. Unfortunately, this experiment has not been confirmed. Attempts to determine whether RNA produced by macrophages was specific for the inducing antigen by hybridization techniques led to conflicting results (11,13). The estimated size of the active RNA is 4S to 10S (5,10,11) which is too small to code for the light chain of the antibody molecule. To date, the messenger hypothesis is far from proven.

Even if the macrophage does not produce a messenger RNA capable of participating in protein synthesis within the antibody-producing cell, it still may play an active role in triggering the immune response. Askonas and Rhodes reported (7) that antigen processing by macrophages enhanced immunogenicity but didn't know whether this function was essential. Frei and coworkers (14) presented good evidence for a key role for the macrophage in the primary immune response. They injected bovine serum albumin into rabbits, waited for phagocytosis to occur, and then collected the serum which contained residual

antigen. When this serum was injected into non-immunized rabbits, most of them did not develop anti-bovine serum antibody. This "filtered" antigen proved to be less immunogenic than an equal amount of "unfiltered" antigen. Gallily and Feldman (1) presented evidence that processing by macrophages was essential. They injected X-irradiated recipients with macrophages which had been allowed to phagocytize Shigella antigen in vitro. The recipients produced agglutinins whereas control mice receiving antigen alone, failed to respond. Macrophages freed of lymphocytes by in vitro culture were effective in restoring the agglutinin response in contrast to lymph node cells which proved to be inactive.

There are also reports in the literature that macrophages depress the immune process (15,16,17) and that they reduce the immunogenicity of soluble antigen (18). However, two of the above studies involved secondary responses (15,16). The single report of a reduction in immunogenicity (18) involved a highly antigenic material administered in nanogram quantities.

Further light on the role of the macrophage in the immune response was shed by the experiments of Nossal and his associates which have been reviewed recently (19). They used radioactive labels to study the fate of antigen injected into rats. When tissue sections of the lymph nodes were examined, it appeared that two kinds of macrophages were involved. Medullary macrophages took up all of the materials injected while the follicular macrophages engulfed only the bacterial antigens. The specificity of follicular localization was confirmed with additional antigens. Weak antigens showed only a trace of

localization in the follicles while non-antigenic substances, with the exception of homologous or autologous globulins, did not localize there at all (20,21). In the spleen, a purposeful movement of antigen-carrying cells, or transfer of antigen from cell to cell was demonstrated resulting in the concentration of antigen in the outer aspects of the germinal centers (2). It has been demonstrated that the capture and retention of these antigens was less efficient in germ-free animals (23), and in those animals subjected to whole body X-irradiation (24), but enhanced in actively or passively immunized animals (25). Localization was increased if antigen was injected as a complex with antibody (26). Particle size alone did not account for the increased localization of immune complexes since heat-denatured human serum albumin was fixed only slightly in the follicles (27). The follicular macrophages appeared to form a fine web of phagocytic reticulum on which antigenic material was retained (28). Electron microscopic autoradiography indicated that the labeled antigen was associated with membrane extensions of the follicular cells. It was difficult to tell whether the antigen was on the cell membrane or on its extensions. The antigen appeared to be "retained" by the follicular macrophages while it was phagocytized by the medullary macrophages (29,30,31).

Opsonins probably have a role in determining the fate of antigen since there is increased follicular localization in the presence of antibody (26). In normal animals, the small lymphocyte probably secretes these opsonins since depletion by thoracic duct drainage (32) or whole body X-irradiation (33) reduced uptake of antigenic material by the follicles. Localization was partially restored by

injection of antibody or by transfusion of normal small lymphocytes.

The preceding experiments provided initial evidence supporting the concept that there was a mechanism for guiding antigen on the intricate path to antibody synthesis, and that two kinds of macrophages were involved. Cohen and coworkers presented conflicting data which indicated that follicular localization was not specific (34). Humphrey and Frank found that soluble protein antigens localized in germinal centers only in the presence of specific antibody (35).

The opsonin macrophage system may be a mechanism for recognizing foreignness, or a mechanism for trapping antigen as long persisting complexes (26,36,37). Since antigen-antibody complexes have improved immunogenicity (37), follicular trapping may enhance antibody formation in the secondary response. The relevance of follicular trapping to the induction process is unproven(31). To complicate the situation, the presence of circulating antibody does not always enhance an immune response. It has been shown to inhibit (38), have a regulatory role (39,40) or depress antibody formation (41,42,43).

If macrophages are essential for processing antigen, the question of control of specificity becomes quite complex. Burnett (44) has suggested that initiation of the immune response results from contact of antigen with antibody-like sites present on the surface of lymphoid cells. The contact stimulates cells to multiply and antibody to be produced. Different clones would have different receptor sites on their surface thus insuring that a certain antigen would stimulate only the right kind and number of cells. He postulated that a secondary response is explained in terms of improved antigen capture due to an

increased number of cells carrying the proper antibody-like site after primary stimulation. Eradication of particular clones in early life results in tolerance. There is some evidence that macrophages recognize foreignness (20) and some claims of selectivity in phagocytosis (45). Evidence to support this idea comes from experiments on reticuloendothelial blockade (45,46). Depletion of opsonins (47) could have been a decisive factor in the apparent specificity of the blockade, but the role of opsonins in blockade is not settled (48).

There have been occasional reports that macrophages themselves can produce antibodies (49,50,51), but these can usually be attributed to failure to obtain a pure macrophage population.

Many interesting observations appear in the literature indicating how the transfer of antigenic material might take place. Sharp and Burwell (52) reported adhesions between macrophages and lymphocytes *in vitro*. Lymphocytic "foot processes" have been suggested as the specialized organelles for performing the attachment (53,54). Clones of lymphocytes surrounding a macrophage were noted by Fishman *et al*. (7). In the electron microscope, direct cytoplasmic connections have been observed between macrophages and lymphoid cells of normal and immunized rabbits (55). Mosier has demonstrated the necessity for cell interaction in continued antibody formation in tissue culture (56). Macrophages appear to be required for blast formation in lymphocyte cultures (57).

Clearcut answers to questions about cell types involved in the immune response depend on improved identification of cell types. Part of the confusion is due to the macrophage-like behavior of lymphocytes

undergoing blast transformation. They stick to glass, phagocytize particulate matter, and bind cytophilic antibody (58,59,60). Unlike macrophages, they are resistant to the cytotoxic action of anti-macrophage serum (58).

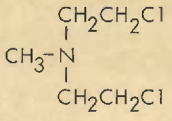
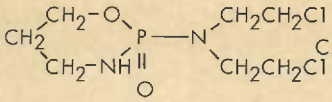
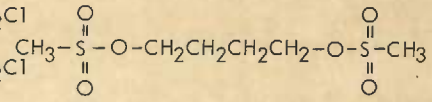
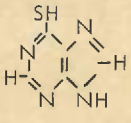
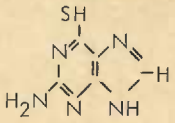
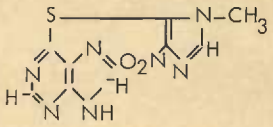
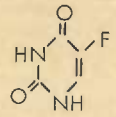
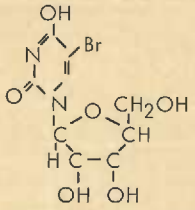
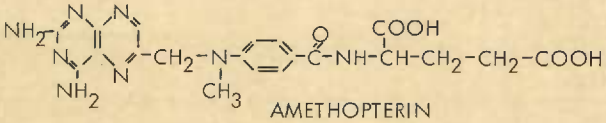
In summary, antigenic material reaching the regional lymph nodes of normal animals is phagocytized by macrophages. Uptake and retention appear to be enhanced by opsonins. Processing is accomplished by selected macrophages which may produce an RNA-antigen complex, endowed with increased immunogenicity. It is not known if processing of antigen by macrophages is an essential step in the induction of the humoral immune response; it appears to be required for particulate antigens.

The activity of X-irradiation (61) and drugs as immunosuppressants (62,63) have recently been reviewed. Their effectiveness is always related to the temporal relationship between their administration and that of antigen. The immune response may be divided into the pre-inductive phase, the time before antigen administration; the inductive phase, occurring between the administration of antigen and the appearance of circulating antibody; and the productive phase, during which antibody is released into the circulation.

The principal classes of immunosuppressive drugs are alkylating agents, purine and pyrimidine analogues, and antagonists of folic acid metabolism. Their biochemical actions have been reviewed (64,65,66, 67). Representative structures of compounds in each of these classes are shown in Table 1 (63).

TABLE 1

Formulas of the principal types of chemical agents used for immunosuppression (63).

TYPE	STRUCTURAL FORMULA
ALKYLATING AGENTS	 NITROGEN MUSTARD  CYCLOPHOSPHAMIDE (CYTOXAN)  BUSULFAN (MYLERAN)
PURINE ANTAGONISTS	 6-MERCAPTOPURINE  6-THIOGUANINE  AZATHIOPRINE (IMURAN)
PYRIMIDINE ANTAGONISTS	 5-FLUOROURACIL  5-BROMODEOXYURIDINE (BUDR)
FOLIC ACID ANALOG	 AMETHOPTERIN

Alkylating agents are believed to interact directly with DNA effecting the proliferation and differentiation of cells. This class of compounds is most effective in the inductive phase of the immune response (63). Cyclophosphamide is an example of the compounds in this class.

The purine analogues (63) act by blocking the interconversion of nucleotides, particularly inosinic acid to adenylic acid. Interference with this central biochemical process leads to widespread intracellular effects, especially on the synthesis of nucleic acids. Imuran (azathioprine) a member of this group, may also bind sulfhydryl groups. These agents are also most effective during the inductive phase of the immune response.

The pyrimidine analogues (63) are specifically incorporated into DNA making it abnormal and incapable of supporting mitosis, thereby stopping cell division. Five-Fluorouracil, a typical representative of this group, is active during the inductive phase of the immune response.

The folic acid antagonists (63) are believed to block the action of dihydrofolic acid reductase, the enzyme required to convert folic acid to its active form, which is required for the metabolism of one-carbon fragments. Usually, this blocks construction of nucleic acids and proteins. Methotrexate, like the other compounds in this group, is effective during the inductive phase of the immune response.

X-irradiation is believed to prevent mitosis by interfering with the buildup of the mitotic apparatus. In bacterial systems, the synthesis of inductive enzymes is inhibited by X-rays (68).

X-irradiation suppresses the immune response best when it is administered during the pre-inductive phase, 24 to 48 hr before antigen administration (70). Evidence that the processing of antigen by the macrophage may be the radiosensitive phase of the primary immune response (1) was recently extended by Kolsch and Mitchison (69). The intracellular fate of phagocytosed antigen in cells from peritoneal exudates of mice was studied using ^{125}I and ^{131}I labeled antigens. After uptake of labeled antigen, the macrophages were homogenized, and the subcellular fractions analyzed by isopycnic centrifugation in sucrose gradients. With normal cells, 90% of the phagocytosed material was degraded within 2 to 3 hr to amino acids while 10% remained intact. Newly phagocytosed antigen was found mainly in the lysosomal turnover compartment of a density of 1.19 g cm^{-3} while antigen which had been in the cell longer was found in a denser storage fraction (1.26 g cm^{-3}) associated with the nuclei. In X-irradiated cells, less antigen was found in the storage compartment. Kolsch and Mitchison postulated that in the X-irradiated macrophages, the observed loss of function might be due to damage to the process which brings antigen into the storage compartment or retains it there for initiating the immune response.

Cytoxan was selected as one of the immunosuppressants used in this project since it was an effective immunosuppressant which destroyed proliferating cells appearing late in the inductive phase of antibody synthesis (71). This transport form of nitrogen mustard (N,N-bis (β-Chloroethyl)-N', O-propylene phosphoric acid ester diamide monohydrate) was synthesized in 1957 (72). Since that time it has been applied

extensively to animal research and has had limited clinical use. It is believed to be converted to the active form in the liver by splitting off the azaphosphohexane group. It may act as a nitrogen mustard or as a powerful phosphorylating agent. It is effective in rabbits, guinea pigs, and mice suppressing not only the initial phases of the immune response, but affecting ongoing antibody synthesis. It has its maximal suppressive effect 24 to 48 hr after antigen administration (71).

Cycloleucine (1-aminocyclopentane-1-carboxylic acid) was a useful immunosuppressant for this project since it is one of the few drugs reported to be effective in the pre-inductive phase of the immune response (73); behaving like X-irradiation. Cycloleucine was first synthesized in 1911 (74) but was not tested for biological activity until the 1950's when it was reported to be an anti-tumor agent (75,76). Amino aciduria results after oral administration in man, but this is probably not responsible for its anti-tumor activity (77). It does not appear to act as an amino acid antagonist in bacterial systems, but antagonizes incorporation of valine into proteins in the rat, possibly by preventing attachment of the amino acid to sRNA (78). Its toxic effects can be prevented by valine in chickens (79) and by methionine and valine in Escherichia coli (80). Cycloleucine does not inhibit enzymatic oxidation or transamination of amino acids (81) and is not incorporated into proteins (82). Its half-life in rats of 20 to 30 days (83) is probably due to extensive renal reabsorbtion and lack of metabolism. It has been shown to suppress experimental allergic encephalomyelitis in rats (84).

Neither methionine, valine nor leucine could prevent this immunosuppression. It is effective in other types of cellular hypersensitivity, and in suppressing the primary immune response, but not the secondary (73). Unlike the purine or folic acid antagonists, it is not toxic to human embryonal fibroblasts in tissue culture. Leukocyte counts are not depressed at therapeutic levels (85). Its mode of action is still unknown.

MATERIALS AND METHODS

Mice: C_3H male and female mice 8 to 10 weeks of age were used in the drug experiments. C57B1/6 mice were employed in the X-irradiation experiment. Animals (Simonsen Laboratories, Gilroy, California) were caged in groups of 6 to 10 and given free access to water and Purina mouse pellets.

Induction and collection of peritoneal exudate cells (86): These are referred to hereafter as macrophages. Three-ml of thioglycollate medium (Difco, Cleveland, Ohio) were injected intraperitoneally into each mouse. Four days later, the mice were killed by cervical dislocation, the peritoneum opened with a Bard-Parker blade, and collection media injected with a plastic syringe fitted with a blunt 18-ga needle. The washings were aspirated and pooled in a plastic centrifuge tube maintained in an ice bath. The cells were centrifuged in the cold (4 C) at 1200 rpm for 10 min and resuspended in Medium 199 at a concentration of $30/10^6$ cells/ml. Cells were mixed in siliconized vials with an equal volume of sheep erythrocyte antigen for injection, intraperitoneally, into recipients. Sterile technique was maintained throughout the procedure.

Collection of spleen cells: Animals were killed by cervical dislocation, the peritoneum opened with a Bard-Parker blade, and the spleen dissected from its surrounding membranes. Three spleens were placed in a Snell cytosieve together with 4.5-ml of Eagle's or Medium 199 and disaggregated by gently pressing them through the stainless steel mesh. The cell suspension was placed in a plastic centrifuge

tube and maintained in an ice bath. The spleen cell suspension was used in this state when Jerne plaque assays were done. For the transfer experiments, the cells were handled in the same manner as the peritoneal exudate suspensions.

Bleeding of animals: Animals were bled from the retro-orbital sinuses 5 and 8 days after antigen administration except as noted in the experimental procedure. Mice were anesthetized, and 0.4 ml of blood was removed from each animal with a glass pipette, and placed in 0.4 ml of citrated saline. The blood was centrifuged at room temperature for 5 min, the serum drawn off, and frozen immediately until assayed.

Hemagglutination assay (87): Serial 2-fold dilutions of individual sera in a volume of 0.2 ml saline were prepared. One drop (approximately 0.05 ml) of a 2% sheep erythrocyte suspension was placed in each tube. After 1 hr at room temperature, the tubes were centrifuged lightly and read immediately by gently tapping each tube. The titer was recorded in $\log_2$ as the last tube with visible agglutination.

Jerne plaque assay (88): This procedure is an assay of anti-sheep erythrocyte antibody at the cellular level. Spleen cells from animals immunized with erythrocytes were suspended in an agar gel with antigen. When antibody was secreted, it was held in place by the agar. When complement was added, the antibody already attached to the erythrocytes lysed them, forming a clear area in the lawn of the antigen. These plaques may be counted in indirect light or stained and counted on a colony counter. The number of plaques/million cells were calculated.

Assay plates were prepared in 2 layers. The base layer, a mixture of 3.3-ml of barbital buffer with phenol red, and 3.3-ml of 2.8% agar was poured into 60 by 15 mm plastic petri dishes, hardened, and refrigerated until used. Sheep RBC (0.05-ml of 10%) and spleen cell suspension (0.05-ml) were added to the agar mixture (0.5-ml of 1.4% melted agar, 0.4-ml 2X Eagle's medium, and 0.05-ml of 1% DEAE dextran) maintained at 45 C, mixed quickly, and spread on top of the base layer. The plates were incubated at 37 C for 1 hr to allow secretion of the antibody. A 10% dilution of guinea pig serum (0.75-ml) in modified barbital buffer was added as a complement source and incubation continued for an additional hour. The complement was poured off and the plates refrigerated overnight for staining and counting the next day. Staining consisted of adding 2 to 5-ml of a stain (10-ml of 2% benzidine in glacial acetic acid, 1.2-ml of 30% hydrogen peroxide, and 90-ml of water) to each plate for 3 to 5 min. Plaques, which appeared as evenly distributed clear areas on a blue background of stained erythrocytes, were counted on a colony counter and the results recorded as plaques/million spleen cells plated.

Drug administration: Drugs were suspended in water immediately before intraperitoneal administration. Cycloleucine (Nutritional Biochemicals Corporation, Cleveland, Ohio) was injected at a dose of 350 mg/kg of mouse 2 days prior to cell transfer. Cytoxan, (Mead-Johnson Laboratories, Evansville, Indiana) was administered at a dose of 200 mg/kg 1 day before cell transfer.

X-irradiation (1): Mice were placed in partitioned lucite boxes

and exposed to total body X-irradiation of 550r (250 kv, 15 ma) at a dose of 37r/min at a target distance of 50 cm.

Sheep erythrocytes preserved in Alsever's solution (Sheep Blood Supply, Portland, Oregon) were washed in 3 vol of sterile physiological saline, centrifuged, and diluted from the packed cell volume. The 2% solutions administered as antigen were made in Medium 199.

Opsonization (89): Mouse anti-sheep RBC serum (0.1-ml titer 1:256) and 25-ml of a 2% suspension of sheep erythrocytes in Medium 199 were incubated at 37 C for 20 min.

In vivo phagocytosis (90): Animals were induced for peritoneal exudate production in the standard manner. Four days later, 0.5-ml of 2% sheep RBCs were injected intraperitoneally and harvested 1 hr later in the usual manner. A Giemsa stain was done on slides prepared from the harvested cells.

Solution for peritoneal exudate cell collection consisted of Hanks' basic salt solution (Microbiological Associates, Albany, California), 1% inactivated calf serum (Microbiological Associates), 10 U of phenol-free heparin/ml (Clay Adams, New York), 100 U of penicillin/ml (Pfzier Laboratories, New York), and 10 mcg of streptomycin/ml (Eli Lilly, Indianapolis, Indiana). The pH was adjusted to 7.1 with 7.5% NaCHO at the time of use.

Cell counts: Nucleated cells in the peritoneal exudate suspensions and splenic suspensions were counted in a Neubauer hemocytometer.

Eagle's media (Hyland Laboratories, Los Angeles, California): This powdered concentrate was hydrated at the time of use and pH adjusted to 7.1 with 7.5% $NaHCO_3$.

Agar was hydrated from Difco agarose.

DEAE-Dextran (Pharmacia, Uppsala, Sweden).

Thioglycollate media was hydrated from Difco thioglycollate powder.

Nembutal for mice was prepared from Nembutal sodium (50 mg/cc, Abbot Laboratories, North Chicago, Illinois), 1.2-ml; ethanol (95%), 1.2-ml; saline, 52.8-ml.

Enzymatic assay for acid phosphatase (91,92): Peritoneal exudate cells were induced by the standard method and collected in cold physiological saline. Spleens were removed and a suspension made with the Snell cytosieve in cold physiological saline. Both cell types were centrifuged in the cold at 1500 rpm for 20 min, washed, and recentrifuged. A dilution of 1:10 of the packed cell volumes, made in saline, were frozen and thawed 5 consecutive times. Extracts were clarified by centrifugation at 2500 g for 20 min at 4 C. For the assay, 0.2-ml of cell extract was added to 1-ml of substrate in a colorimeter tube and incubated at 38 C for 30 min. Finally, 3-ml of 0.1 N NaOH was added to develop color and stop the reaction, and the tube read at 410 mμ on a Beckman Spectrophotometer 20. Substrate and serum blanks were handled in the same manner and subtracted from the unknown before calculation. Results were recorded as units of enzymatic activity/ml of packed cells. A unit was defined as the amount of enzyme required to release 0.5 mM of p-nitro-phenol/liter as determined from a standard curve.

Gomori stain for acid phosphatase (93): Smears of peritoneal exudate cells were made on acid-cleaned slides, air dried, fixed in cold buffered formalin for 1 hr at 4 C, washed with distilled water,

and placed in substrate for 4 hr at 37 C. Slides were washed with distilled water, placed in diluted ammonium sulfide for 2 min, rewashed, and counter-stained with safranin. They were examined under the oil immersion lens.

RESULTS

The first experiment was an attempt to repeat the work of Gallily and Feldman (1) using normal C57B1/6 macrophages injected together with antigen into previously irradiated recipients. Measurement of antibody was limited to the plaque assay procedure since the serum of this strain characteristically hemagglutinated sheep erythrocytes to a high titer, but hemolytic plaque-forming cells were absent from their spleens. Two days after X-irradiation, 1 group of mice received normal macrophages and antigen, a second was given an equal number of spleen cells and antigen, while a third group received antigen alone. The non-irradiated controls were also injected with sheep erythrocytes. For Jerne assays, performed 5 days after cell transfer, each splenic suspension was plated in triplicate; the mean value is recorded in Table 2 as plaques/million cells. Macrophage-antigen mixtures elicited about 20 times as much antibody as antigen alone in X-irradiated recipients, while spleen cell-antigen mixtures caused no augmentation of antibody formation. These results confirmed those of Gallily and Feldman (1) and encouraged further work. Subsequent experiments utilized 10-week-old male and female C_3H mice treated with the immunosuppressive drugs, cycloleucine or Cytoxan.

The objective of the next set of experiments was to determine whether macrophages from normal or Cytoxan-treated donors could elicit antibody formation in cycloleucine-suppressed recipients. One group received normal macrophages and antigen, a second received macrophages from Cytoxan-treated donors and antigen, while the control group was

Table 2

Antibody Production by Irradiated C57B1/6 Mice Following Inoculation of Macrophages or Spleen Cells

Irradiated Recipient	Plaque-Forming Units/10^6 Spleen Cells*
Normal macrophages + sheep RBCs	157
Normal spleen cells + sheep RBCs	4
Sheep RBCs alone	8
Non-irradiated	
Sheep RBCs	492

*Each value represents triplicate counts from a pool of three spleens.

Table 3

Increased Antibody Production in Cycloleucine-Suppressed C_3H Mice by Macrophage-Antigen Mixtures

Suppressed Recipients	No. Expts.	Hemagglutinin Titer after 5 Days: No. Mice	5 Days: Mean Titer Log_2	5 Days: S.E.	8 Days: No. Mice	8 Days: Mean Titer Log_2	8 Days: S.E.
Normal macrophages + sheep RBCs	3	21	5[a]	0.27	18	5.4[c]	0.38
Cytoxan macrophages* + sheep RBCs	1	5	4.8	0.66	2	7.5	0.5
Sheep RBCs alone	3	20	2.3[b]	0.27	19	3.7[d]	0.26
Non-suppressed Control							
Sheep RBCs alone	1	10	7.8	0.20	10	8	0.21

a & b significant at 0.001 level

c & d significant at 0.01 level

*From donors given 200 mg/kg of Cytoxan 1 day prior to transfer.

given antigen alone. Each animal was bled from the orbital sinus at 5 and again at 8 days after antigen administration. Individual hemagglutinin titrations were performed and the mean value to the $\log_2$ determined. The results of 3 separate experiments, compatible by t test, were pooled and summarized in Table 3. After 5 days, the hemagglutinin titers in mice given normal or Cytoxan macrophages and antigen were significantly elevated over that observed in the control group given antigen alone. The 8-day titers followed the same pattern, but the data were less significant. Of special interest was the fact that the alkylating agent, Cytoxan, did not appear to inhibit the antigen-handling function of the macrophage.

The third set of experiments tested the ability of macrophages or spleen cells from normal donors, injected with antigen, to elicit a hemagglutination response in Cytoxan-treated recipients. For this purpose, 200 mg/kg of Cytoxan, a dose adequate to inhibit the immune response (71), was administered to all recipients 1 day prior to antigen administration and cell transfer. The first group was injected with normal macrophages and antigen, the second received spleen cells and antigen, while the third group received antigen alone. Each animal was bled from the orbital sinus at 5 and again at 8 days after cell transfer. Individual hemagglutinin titrations were performed and the mean value to the $\log_2$ determined. Three separate experiments, compatible by t test, were pooled and summarized in Table 4. The data indicate that hemagglutinin production in the Cytoxan-suppressed mice was not augmented by supplying normal macrophages. Normal spleen cells, as previously reported by Santos for rats (94), did elicit an immune

Table 4

The Effect of Macrophages and Spleen Cells from Normal Donors on the Production of Antibodies in Cytoxan-Suppressed Recipients

		Hemagglutinin Titer after					
		5 Days			8 Days		
Suppressed Recipients	No. Expts.	No. Mice	Mean Titer Log_2	S.E.	No. Mice	Mean Titer Log_2	S.E.
Normal macrophages + sheep RBCs	3	17	1.9[a]	0.19	15	1.5[c]	0.25
Spleen cells + sheep RBCs	1	5	4.4	0.21	4	4.3	0.25
Sheep RBCs alone	3	18	1.5[b]	0.26	15	2.3[d]	0.29

a & b not significant.

c & d significant at 0.1 level.

response in the Cytoxan-suppressed mice. It would appear, therefore, that the defect produced by Cytoxan was directed against cells other than macrophages.

An additional experiment was designed to determine if macrophages from cycloleucine-treated donors were less effective than normal macrophages in stimulating an antibody response in cycloleucine-suppressed recipients. Cycloleucine was administered to some donors and all recipients 2 days prior to cell transfer. One group of recipients received macrophages from cycloleucine-treated donors and antigen, a second group received a normal macrophage-antigen mixture, and another group a normal spleen cell-antigen mixture, while the control group was injected with sheep erythrocytes alone. A non-suppressed control received sheep erythrocytes. Each animal was bled from the orbital sinus 5 days after cell transfer, and individual hemagglutinin titers determined. At this time, 3 animals from each group were selected for a Jerne plaque assay. Each splenic suspension was plated in triplicate, the mean value determined, and the plaques/million nucleated cells plated was recorded in Table 5. Transfer of macrophages from cycloleucine-treated donors transferred with antigen to cycloleucine-treated recipients did not elicit the characteristic antibody increase produced by the transfer of a normal macrophage-antigen mixture. As expected, the spleen cell and antigen controls were also ineffective. These data tend to localize cycloleucine activity to the macrophage.

As indicated in the introduction, defining a macrophage on a morphological basis was a difficult task. Ordinarily, it was characterized as a large monocytic cell possessing many lysosomes and

Table 5

Antibody Production in Cycloleucine-Suppressed Recipients; Effect of Macrophages from Cycloleucine-Treated C_3H Mice

Suppressed Recipients	No. Mice	Mean Titer Log_2	S.E.	Number of Plaque-Forming Units/10^6 Spleen Cells
Cycloleucine macrophages + sheep RBCs	10	3.0[c]	0.36	2.66
Normal macrophages + sheep RBCs	10	6.1[a]	0.23	11.33
Normal spleen cells + sheep RBCs	10	3.1[d]	0.28	5.95
Sheep RBCs alone	10	2.3[b]	0.36	0.58
Normal Recipients				
Sheep RBCs alone	10	7.8[e]	0.20	87.5

a & b, a & c, a & d, a & e significant at 0.001 level.

b & c not significant, difference between b & d not significant.

b & e significant at 0.001 level.

capable of a high level of phagocytosis. On examination of the Giemsa-stained slides prepared from the induced macrophage exudate, 80 to 90% of the population appeared to be of this cell type. The first photomicrograph demonstrates typically large macrophages with many vacuoles and prominent nuclei.

To be certain that this cell type was capable of active phagocytosis, an in vivo experiment was done in which sheep erythrocytes were injected into the peritoneal cavity of induced mice, and the exudate harvested after 1 hr. A typical Giemsa stained field of such a preparation is shown in the second photomicrograph. The macrophages are stuffed with engulfed erythrocytes indicating that they are able to take up large numbers of foreign cells.

A series of Gomori stains for acid phosphatase were done to demonstrate that these peritoneal exudate cells had many lysosomes. A typical field, shown in the last photomicrograph, indicates that these cells have many lysosomes, evidenced by heavy staining of the enzymatic product.

An experiment in which the levels of acid phosphatase activity in peritoneal exudate cells and splenic cells were compared is recorded in Table 6. The peritoneal exudate population demonstrated a much higher level of acid phosphatase activity than spleen cells.

The next experiment (Table 7) produced evidence that macrophages from cycloleucine-treated animals were as capable of phagocytizing antigens as normal macrophages. One group of animals was treated with cycloleucine 2 days before the administration of sheep erythrocytes. Antigen was administered to normal animals and to cycloleucine-treated

Plate 1

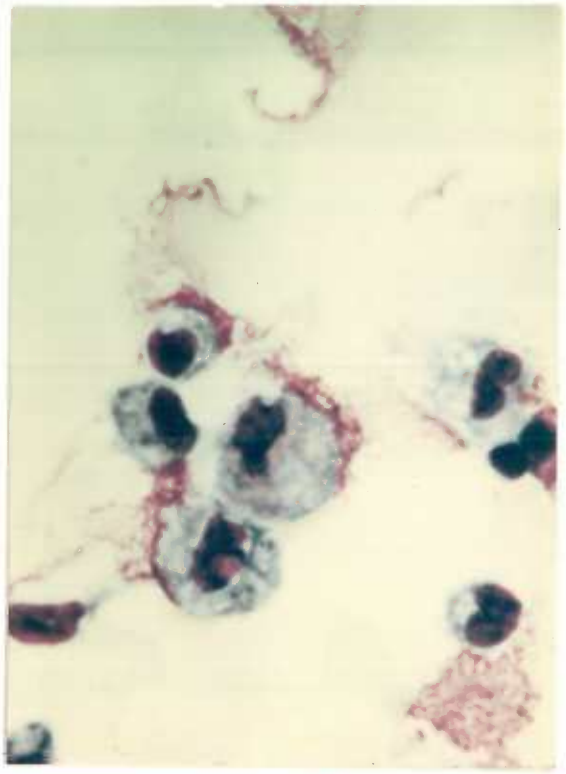
Giemsa stain of a typical macrophage population from mouse peritoneal cavity. (100 X)

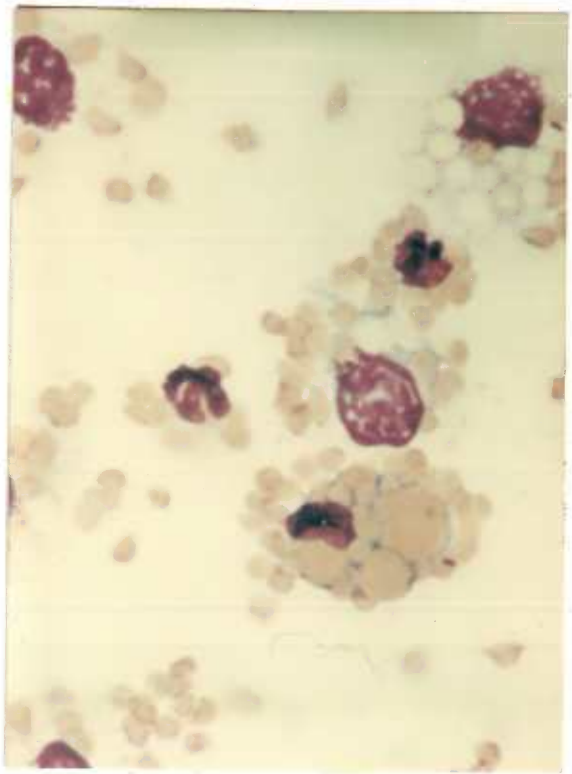
Plate 2

Mouse peritoneal macrophages which have phagocytized sheep RBC antigen *in vivo*.

Plate 3

Gomori stain for acid phosphatase of typical mouse peritoneal macrophages.





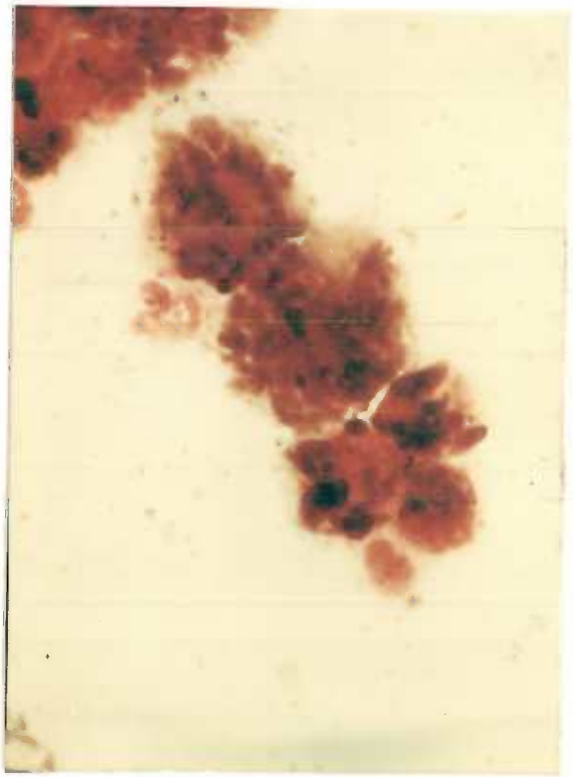


Table 6

Enzymatic Assay of Acid Phosphatase Level in Induced Macrophages Compared to the Level in Spleen Cells of Mice

Cell Type	Units of Enzymatic Activity/ml of Packed Cells
Macrophage	163
Spleen cells	39

Table 7

Phagocytosis of Sheep Erythrocytes by Normal Macrophages and the Macrophages of Cycloleucine-Treated Animals Performed In Vivo

Cell Type	No. Cells Counted	% Cells Containing 5 or More Sheep RBCs
Normal macrophages	2,000	80
Macrophages of a cycloleucine-treated animal	2,000	75

animals at the same time. After 1 hr, both macrophage populations were harvested, and slide preparations stained with Giemsa. The findings indicate that cycloleucine effects an intracellular stage of antigen processing.

DISCUSSION

The confirmation of the experiments of Gallily and Feldman (1) provides additional evidence favoring an active role for the macrophage in the primary immune response. It has permitted use of a similar cell transfer system in which chemical immunosuppressants were substituted for X-irradiation and sheep erythrocytes for Shigella antigen. In vitro phagocytosis proved unsuccessful with this large antigen so it was administered to the recipients with the transferred cells. This method has provided information regarding the selective susceptibility of cell types to immunosuppressive drugs and the role of these cell types in antibody synthesis. Cycloleucine proved to be an especially useful tool for studying the role of the macrophage. The ability of macrophage-antigen mixtures to trigger antibody production in cycloleucine-suppressed recipients was demonstrated. The inability of macrophages from cycloleucine-treated donors to elicit antibody formation in cycloleucine-suppressed recipients helped establish which cell type was most susceptible to this drug. The latter experiment was difficult to perform due to the added toxicity which resulted in a high mortality rate among recipients. Spleen cell controls were included in these experiments to rule out the possibility of antibody formation by lymphocytes present in the peritoneal exudates. Although these controls received 10 times as many lymphocytes as test animals, antibody production was not augmented. Spleens were employed as a source of lymphocytes rather than the thoracic duct or lymph nodes due to technical difficulties in maintaining cell viability during handling.

The difference in titers between the suppressed animals who received macrophages and the non-suppressed controls could have been due to the effect of residual drugs on transferred cells. The long half-life of cycloleucine could have prevented the high level of agglutinin formation reported for X-irradiated recipients (1).

Experiments in which recipient mice were suppressed with Cytoxan confirmed the belief that the macrophage was not the cell most susceptible to this drug. The splenic population contained susceptible cells as evidenced by antibody formation in suppressed animals receiving them. Macrophages from Cytoxan-treated donors transferred with antigen to cycloleucine-suppressed recipients remained competent indicating that they were still capable of processing or storing antigen.

Cycloleucine appears to mimic X-irradiation in its temporal relation to antigen administration (70,95), its target cell, and its intracellular site of action (1). Both agents may affect other cell types (85,95) but in the primary immune response, there are strong similarities. It would be interesting to repeat the experiment of Kolsch and Mitchison (69) with drug-treated macrophages since cycloleucine, a particularly good chelating agent, could interfere with the intracellular transport or storage of immunogenic antigen.

The possible role for macrophages in the humoral immune response is worthy of speculation. They could produce mRNA capable of entering immunocompetent cells and coding for antibody synthesis. This would complicate the recognition mechanism since one site would be required for antigen recognition and another for attachment to a competent lymphocyte. Failure to obtain antigen-free RNA preparations and the

general lack of specificity among macrophages detracts from the feasibility of this model. A second possibility is that antigen may be rendered more immunogenic by complexing to non-messenger RNA. If the RNA in the complex is the same for all antigens, it could represent a mechanism for storage of the "immunogenic" antigen, or for facilitating binding to or entry into immunocompetent cells. Finally, the macrophage could provide sustained release of antigen in the most immunogenic quantities and sizes. Mosier's observation that cell interaction is required for continued antibody synthesis (56) makes this a feasible model. Inability to provide sustained antigen release could explain the failure of X-irradiated and cycloleucine-treated macrophages to trigger the primary immune response.

The evidence supporting a vital role for the macrophage in antibody synthesis was gathered in systems employing the primary immune response while the negative evidence came from systems in which a secondary response was measured. For particulate antigens, the macrophage is almost certainly required to trigger the primary immune response. This may be due to a necessity for efficient handling of antigen in the induction of the primary response since a limited number of cells are involved. After cell replication begins, this frugality is probably less essential resulting in continued but non-essential function for the macrophage in the secondary immune response. If this is the case, many of the conflicts in the literature would be explained.

SUMMARY AND CONCLUSIONS

The role of the macrophage in the primary immune response was studied utilizing the method of cell transfer to drug-suppressed mice. Cycloleucine, a newly described immunosuppressive drug, appears to affect the ability of the macrophage to process or store antigen during the primary immune response to sheep erythrocytes. It does not seem to interfere with antigen uptake, indicating an intracellular site of action. Normal macrophages transferred with antigen to cycloleucine-suppressed recipients triggered antibody formation while normal spleen cells or macrophages from cycloleucine-treated donors failed to do so. Cytoxan, conversly, did not inhibit macrophage activity but did affect cells from the spleen.

The principle findings of this thesis provide additional evidence in favor of a processing or storage role for the macrophage in the primary immune response and demonstrate that cycloleucine mimics the action of X-irradiation on the macrophage.

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APPENDIX

Medium

Medium 199 without bicarbonate (96,97)

Amino Acids

Components	mg/liter	Components	mg/liter
L-Alanine	25.0	L-Leucine	60.0
L-Arginine HCL	70.0	L-Lysine HCL	70.0
L-Aspartic acid	30.0	L-Methionine	15.0
L-Cysteine HCL	0.1	L-Phenylalanine	25.0
L-Cystine	20.0	L-Proline	40.0
L-Glutamic acid	67.0	L-Serine	25.0
L-Glutamine	100.0	L-Threonine	30.0
L-Glycine	50.0	L-Tryptophan	10.0
L-Histidine HCL·H_2O	22.0	L-Tyrosine	40.0
L-Hydrosyproline	10.0	L-Valine	25.0

Vitamins

P-Aminobenzoic acid	0.050	Nicotinamide	0.025
Ascorbic Acid	0.050	Nicotinic Acid	0.025
D-Biotin	0.010	Pyridoxal HCL	0.025
Calciferol	0.100	Pyridoxine HCL	0.025
D-Ca-Pantothenate	0.010	Riboflavin	0.010
Cholesterol	0.200	Thiamine HCL	0.010
Choline Chloride	0.500	DL-Tocopherolphosphate (Na_2)	0.010
Folic Acid	0.010	Tween 80*	5.000
i-Inositol	0.050	Vitamin A	0.100
Menadione	0.010		

Other Components

Adenine HCL·H_2O	12.10	L-Glutathione	0.05
Adenosine-5'-Monophosphoric acid, dihydrate (AMP) (Muscle Adenylic Acid)	0.20	Guanine HCL·H_2O	0.33
		Hypoxanthine	0.30
		Phenol Red	20.00
Adenosine-5'-Triphosphate disodium, tetrahydrate (ATP)	1.00	Ribose	0.50
		Sodium Acetate $3H_2O$	83.00
		Thymine	0.30
Deoxyribose	0.50	Uracil	0.30
Dextrose	1000.00	Xanthine	0.30

*Trademark of Atlas Powder Company

Inorganic Salts

Components	mg/liter	Components	mg/liter
$CaCl_2 \cdot H_2O$	186.0	$MgSO_4 \cdot 7H_2O$	100.0
$Fe(NO_3)_3 \cdot 9H\ O$	0.7	NaCl	8000.0
KCl	400.0	$NaHCO_3$	1250.0
KH_2PO_4	60.0	$Na_2HPO_4 \cdot 7H_2O$	94.0
$MgCl_2 \cdot 6H_2O$	100.0		

Alsever's Solution

Dextrose	20,500	NaCl	4200.0
		Sodium Citrate	8000.0

Hanks' Balanced Salt Solution

NaCl	8000.0	$Na_2HPO_4 \cdot 7H_2O$	90.0
KCl	400.0	KH_2PO_4	60.0
$CaCl_2 \cdot H_2O$	186.0	Dextrose	1000.0
$MgSO_4 \cdot 7H_2O$	100.0	Phenol Red	20.0
$MgCl_2 \cdot 6H_2O$	100.0	$NaHCO_3$	350.0

Eagle's Basal Medium (98,99)

Amino Acids		Inorganic Salts and other Components	
L-Arginine HCl	21.1	$CaCl_2 \cdot 2H_2O$	186.0
L-Cystine	12.0	KCl	400.0
L-Glutamine	292.0	KH_2PO_4	60.0
L-Histidine $HCl \cdot H_2O$	10.5	$MgCl_2 \cdot 6H_2O$	100.0
L-Isoleucine	26.2	$MgSO_4 \cdot 7H_2O$	100.0
L-Leucine	26.2	NaCl	8000.0
L-Lysine HCl	36.5	$NaHCO_3$	350.0
L-Methionine	7.5	$Na_2HPO_4 \cdot 7H_2O$	90.0
L-Phenylalanine	16.5	Dextrose	1000.0
L-Threonine	23.8	Phenol Red	20.0
L-Tryptophan	4.0		
L-Tyrosine	18.1		
L-Valine	23.4		
Vitamins		**Vitamins**	
D-Biotin	1.0	Nicotinamide	1.0
D-Ca-Pantothenate	1.0	Pyridoxal HCl	1.0
Choline Chloride	1.0	Riboflavin	0.1
Folic Acid	1.0	Thiamine HCl	1.0
i-Inositol	1.8		

Barbital Buffer
(100)
4X maintained as a stock solution

5,5-Diethylbarbituric acid (Barbital	2.8750 gm
Sodium 5,5 diethylbarbiturate (Barbital sodium)	1.8750 gm
Calcium chloride·$2H_2O$	.1103 gm
Magnesium chloride·$6H_2O$	.5083 gm
Sodium chloride	42.5000 gm
Distilled water to 1 liter	

Stains

Giemsa's stain (101)

(a) Stock solution
Giemsa powder, 0.3 gm
Glycerin, 25.0 cc
Methyl alcohol (absolute), 25.0 cc

(b) Stain was diluted by adding 1 cc of stock solution to 10 cc of distilled water.

(c) Procedure:
(1) The smear was fixed with methyl alcohol for 3 to 5 min (in Coplin jar);
(2) dried in the air;
(3) immersed in the diluted stain for 20 to 30 min (in Coplin jar);
(4) washed with distilled water;
(5) stood on end to dry;
(6) and examined under oil immersion.

Wright's stain (101)

(a) Stock solution
Wright's powder, 0.3 gm
Glycerin, 3.0 cc
Methyl alcohol, 97.0 cc

(b) Stain was mixed with a mortar and put into a bottle to stand overnight; filtered and allowed to stand a few days before using.

(c) Buffer solution
Potassium phosphate (monobasic), 1.63 gm
Dibasic sodium phosphate, 3.2 gm
Distilled water, 1000 cc

(d) Procedure
(1) The dried smear was covered completely with stain for 1 to 3 min;
(2) buffer solution was added to the stain on the smear drop by drop until a greenish, metallic scum appeared on the surface. The quantity of solution completely flooded the smear without running over the edges and left for 3 min.
(3) The stain was then washed off with water;
(4) dried standing on end;
(5) and examined with the oil immersion lens.

Leukocyte diluting fluid consisted of 1 cc glacial acetic acid and 1 cc of 1% aqueous solution of gentian violet in 100 cc of distilled water.

Reagents for Acid Phosphatase Assay (91,92)

Reagent A, Sorenson's M/10 citrate-HCL buffer, pH 4.8.
21.008 gm citric acid was dissolved in a liter flask; 200 ml of 1 N NaOH and water added to 1 liter. 100 ml of N/10 HCL was added to 900 ml of the above solution.

Reagent B, 0.8% solution of p-nitrophenyl phosphate in .001 N HCL.

Equal parts of reagents A and B served as the substrate, refrigerated until used.

Reagents for Gomori Stain for Acid Phosphatase (93)

Substrate: .05 M acetate buffer, pH 5, 30 cc
.8% Sodium Beta Glycerophosphate, 3 cc
Lead nitrate, 36 mg
Fixative: Neutral Buffered Formalin
37-40% Formalin, 50 cc
Water, 450 cc
$NaH_2PO_4 \cdot H_2O$, 2 gm
Na_2HPO_4