CELL-MEDIATED IMMUNE RESPONSE IN VITRO*

III. The Requirement for Macrophages in Cytotoxic Reactions against Cell-Bound and Subcellular Alloantigens

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The combined use of in vitro culture techniques together with efficient cell separation methods have revealed that macrophages are essential participants in the initiation of some antibody responses (1–4). The experimental design used was to obtain "purified" (macrophage-depleted) lymphocytes by a cell separation procedure and to demonstrate that these cells could not respond to a particular antigen unless macrophages were also present. Several mechanisms of macrophage function during the induction of the humoral immune response have been proposed. Since it was found that in vitro responses to particulate antigens, such as whole erythrocytes, required the participation of macorphages, whereas responses to smaller sized antigens, such as "soluble sheep red blood cell antigen" (3), or polymeric flagellin of *Salmonella adelaide* (POL)[1] did not, it was proposed that the role of macrophages may therefore be merely to reduce antigenic particles to a smaller and more immunogenic size. Experiments demonstrating that macrophage supernatants enhance responses to erythrocytes (5, 6) are consistent with this simple mechanism of macrophage function. Recently it was found that the immune response to thymus-dependent antigens, even those of small size, such as monomeric flagellin (MON), dinitrophenylated MON (DNP MON), or DNP fowl gamma globulin (DNP FγG), all required the presence of macrophages, whereas responses to the same antigenic determinants (DNP) on polymeric carriers (i.e. POL or DNP POL) which are thymus independent were also found to be

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[1] *Abbreviations used in this paper*: AFC, antibody-forming cells; AMS, rabbit anti-mouse macrophage serum; CL, cytotoxic lymphocytes; CMI, cell-mediated immune response; DNP, dinitrophenol; DNP FγG, dinitrophenylated fowl gamma globulin; DNP MON, dinitrophenylated monomeric flagellin; DNP POL, dinitrophenylated polymeric flagellin; DPM, disintegrations per minute; FCS, fetal calf serum; FEM, fortified Eagle's medium; MLC, mixed lymphocyte culture; MON, monomeric flagellin; NRS, normal rabbit serum; PEC, peritoneal exudate cells; POL, polymeric flagellin of *Salmonella adelaide*; SRC, sheep red blood cells.

macrophage independent (7). It was suggested that macrophages also participate in the process of T-B lymphocyte cooperation (7).

Less is known about the role of macrophages in cell-mediated cytotoxic responses to cell surface alloantigens. These immune responses only involve T lymphocytes, both in vivo (8, 9) and in vitro (10). The mitotic response to alloantigens, the mixed lymphocyte culture (MLC), has been found to be macrophage dependent (11–13). Since the MLC is considered the antigen recognition and proliferative phase of a cell-mediated immune response (CMI), a critical role of macrophages in CMI has thus been proposed. This communication investigated the role of macrophages in an in vitro allograft model for the generation of cytotoxic lymphocytes, based on a "one way" mouse MLC (14). An efficient, surface adherence cell separation method was combined with the use of specific rabbit anti-mouse macrophage serum to obtain populations of mouse lymphocytes devoid of phagocytic activity. The capacity of these functionally pure lymphocyte populations to respond against both cell-bound and subcellular H-2 alloantigens was investigated. The results presented here establish that macrophages are essential for the cell-mediated cytotoxic response in vitro.

Materials and Methods

Mice.—Female CBA/H/Wehi and BALB/c mice aged more than 60 days were used throughout.

Antigens.—Sheep red blood cells (SRC) were collected into Alsever's solution, kept for at least 1 wk at 4°C, then washed three times in 0.9% saline just before use. Usually 3×10^6 cells were used per culture. Dinitrophenylated polymeric flagellin (DNP POL) was prepared as described elsewhere (15). The average DNP substitution per monomeric unit in the single batch used was 1.5 DNP groups. Subcellular H-2^d antigen was prepared as reported for HL-A antigens (16) by papain digestion and KCL extraction of the residuum from membranes of P-815X-2 (DBA/2, H-2^d) tumor cells. Its preparation and immunological properties are described in detail elsewhere.[2]

Treatment with AKR Anti-ΘC3H Serum.—Anti-ΘC3H serum was raised in AKR/J mice by the method of Reif and Allen (17). After inactivation at 56°C for 30 min, its potency was tested by cytotoxicity against CBA thoracic duct lymphocytes (18). Anti-Θ serum was used together with agarose-absorbed guinea pig complement under optimal conditions for killing T cells as described elsewhere (10).

Peritoneal Exudate Cells.—CBA mice of 6–9 months of age were injected intraperitoneally with 1 ml of sterile proteose peptone broth (Difco Laboratories, Inc., Detroit, Mich.). 4 days later, the peritoneal exudate cells (PEC) were harvested into phosphate-buffered saline containing 100 μg/ml of streptomycin and 100 units/ml of penicillin G and were washed three times through fetal calf serum (FCS) before use.

Cell Separation Methods.—Of the various methods for the separation of mouse phagocytic cells from nonphagocytic cells (18, 19, 4), the active adherence method of Shortman et al. (20) seems the most efficient. Mouse spleen cells populations can be fractionated to a lymphocyte preparation extensively depleted of phagocytic cells, yet with a relatively high lympho-

[2] Wagner, H., W. Boyle, and Marc Feldmann. Cytotoxic allograft reaction induced by subcellular antigens. Manuscript in preparation.

cyte recovery. Mouse spleen cells were passed through a large (3 × 18 cm) column of large (300–600 μ) siliconized glass beads at 37°C in medium 199 (Commonwealth Serum Laboratories, Melbourne, Australia) containing 50% isologous mouse serum. The cells which passed through the column were termed purified lymphocytes and were essentially devoid of functional phagocytic cells (21). The recovery of purified lymphocytes was usually between 35 to 45% of the viable lymphocyte input.

Anti-Macrophage Serum.—Rabbit anti-mouse macrophage serum (AMS) used for two of the experiments was kindly donated by Dr. K. Shortman and was prepared as reported elsewhere (5). The other experiments were performed with anti-macrophage serum prepared and made macrophage specific as described by Feldmann and Palmer (3). AMS prepared according to this protocol has been shown not to be cytotoxic for both T and B lymphocytes (22). The specificity of the AMS used was tested before use in the allograft system by testing its effect on the humoral responses against whole SRC and the macrophage-independent antigen DNP POL (2). When it was used at a final dilution of 20% in the culture medium for the 4 day culture period, the number of antibody-forming cells (AFC) against SRC was markedly reduced whereas the number of AFC against DNP POL remained virtually unchanged (Table I). The normal rabbit serum (NRS) was subjected to the same absorption procedures as the AMS (3). Antigen concentration per culture was 3×10^6 SRC and 0.1 μg of DNP POL.

TABLE I

Specificity of the Rabbit Anti-Macrophage Serum

Serum	Antibody response	
	SRC	DNP
	AFC/culture ± SE	
20% NRS	3960 ± 980	345 ± 100
20% AMS	348 ± 34	355 ± 89

Cell-Culture.—

Humoral response against SRC and DNP POL: Spleen cells and purified lymphocytes were cultured in a modification of the Marbrook-Diener system (23) as recently described (24). Eagle's minimal essential medium with supplementary nonessential amino acids was obtained from Grand Island Biological Co., Grand Island, N. Y. This was supplemented with 5% FCS (Commonwealth Serum Laboratories), 100 μg/ml of streptomycin, 100 units/ml of penicillin G, and was buffered with sodium bicarbonate. Cultures were placed in a humidified incubator at 37°C in an atmosphere of 10% CO_2 in air usually for 3.7 days. All cultures were set up in triplicate.

Allograft response against H-2^d antigens: The culture system used was based on that of Marbrook and Diener as described in detail by Wagner and Feldmann (14). In brief, 60×10^6 CBA spleen cells (H-2^k) were cultured together with 15×10^6 mitomycin C-treated BALB/c spleen cells (H-2^d). Mitomycin C (Sigma Chemical Co., St. Louis, Mo.) treatment was performed at a final concentration of 40 μg/ml at 37°C for 30 min, and the cells were washed twice through FCS. The cell suspensions were placed in a glass tube of a diameter of 2.1 cm, sealed off by a dialysis membrane, and suspended from the stopper of a 125 ml Erlenmeyer flask containing tissue culture medium. The cultures were usually terminated at day 6, which was the optimum for the generation of cytotoxic activity in the system used (14). The viability of the spleen cells after 6 days was 10–15%. Cultures were set up in triplicate.

Mixed lymphocyte culture: The MLC was performed in the same culture flasks as used for the humoral response, with a glass tube of 1.1 cm diameter. To ensure comparable culture

conditions in both systems the cell density per surface area was kept constant. Only 12×10^6 CBA spleen cells were cultured together with 4×10^6 mitomycin C-treated allogeneic BALB/c spleen cells in the smaller glass tubes. At day 4, the time of the optimal blastogenic response (14), the cells were pulsed with thymidine-^{3}H (Radiochemical Centre, Amersham, England) for 7 hr at a final concentration of 0.7 μCi/ml, and the acid-precipitable radioactivity determined according to the method of Wilson (25) as described in detail previously (14). Counts per minute were converted to disintegrations per minute (DPM) using external standard for quench correction (14). The stimulation index compares the ratio of thymidine-^{3}H incorporation of the allogeneic combination with that of the controls (thymidine-^{3}H uptake of 12×10^6 reactive lymphocytes and that of 4×10^6 mitomycin C-treated BALB/c spleen cells).

Enumeration of Antibody-Forming Cells.—Cells forming antibody (AFC) were detected by Cunningham and Szenberg's modification (26) of the hemolytic plaque technique. The number of AFC to the DNP determinant were determined as described elsewhere (15).

Cell-Mediated Cytotoxicity Assay.—^{51}Cr assay is a modification of that described by Brunner et al. (27) and was performed as described previously in detail (14, 10).

Labeling of target cells: As BALB/c (H-2^d) and DBA/2 (H-2^d) mice are of identical H-2 specificity, the DBA/2-derived mastocytoma line P-815X-2 were used as target cells in the cytotoxicity assays. This cell line was kept in continuous exponential culture by Dr. Alan W. Harris. Usually $3–4 \times 10^6$ cells were labeled with 100 μCi of chromate-^{51}Cr (CEA, Gif-Sur-Yvette, France) in a final volume of 1 ml of fortified Eagle's medium ("FEM," Grand Island Biological Co.) for 20 min at 37°C. The cells were washed twice through FCS and adjusted to a concentration of 10^5 cells per 100 μl.

Assay: At day 6 of culture, the cells from triplicate cultures were harvested, pooled, and washed twice. The viability of the cultured cells in the positive control (unfractionated CBA spleen cells together with unfractionated mitomycin C-treated BALB/c spleen cells) was determined by the eosin dye exclusion method (28) and the cell concentration adjusted to a 5×10^5 viable lymphocytes/ml. Usually a dilution of 1 to 4, 1 to 16, and 1 to 64 of these cells was assayed in FEM supplemented with 10% heat-inactivated FCS. Similarly the cultures derived of purified lymphocytes were harvested, pooled, and adjusted to the same volume as the positive control and diluted in equal manner in order to correlate the response to that of "unfractionated" cells. 1 ml each of the specific cell dilutions was pipetted to 35 × 10-mm Petri dishes (Falcon Plastics, Div. B-D Laboratories, Inc., Los Angeles), and 50 μl of target cells (5×10^4 cells) were added, thus resulting in a ratio of cultured viable cell to ^{51}Cr-labeled target cells of 100 to 1, 25 to 1, 6.1 to 1, and 1.8 to 1. Each assay was performed in triplicate. The dishes were placed in an airtight box, gassed with 10% CO_2, and rocked on a platform for 200 min. The cells were then harvested, transferred into plastic tubes, a drop of 5% SRC solution added, centrifuged, and the supernatant separated from the pellet. The radioactivities of the supernatant and pellet were determined in an automatic well-type radiation counter and the results expressed as per cent of maximal ^{51}Cr release as determined by freezing and thawing 5×10^4 labeled cells four times. The results in the tables and figures are expressed as the specific lysis which is corrected for background lysis. The background lysis, in the presence of normal unstimulated spleen cells, was always in the range of 8–13% after 200 min of incubation except for the experiments with subcellular H-2^d antigens. In these experiments, the ^{51}Cr test was extended to 6 hr, thus raising the background lysis up to 15–20%.

RESULTS

The Cell-Mediated Response of Macrophage-Depleted Spleen Cells.—To investigate the role of macrophages in an allograft response in vitro portions of pools of CBA (H-2^k) and BALB/c (H-2^d) spleen cells were each depleted of

macrophages by the adherence column method of Shortman et al. (21). The efficiency of the cell separation procedure was established by comparing the antibody responses of the fractionated and unfractionated CBA spleen cells to SRC, and to DNP POL, and also the mitotic response (MLC) to BALB/c cells. The known macrophage dependence of both the MLC (11–13) and the SRC response (1–4) was used as an index of the efficiency of macrophage depletion, and the normal response to DNP POL, previously shown to be macrophage independent (2, 3), was used as a criterion of the functional integrity of the fractionated cells. Table II illustrates a representative experiment incorporating these controls. The three types of culture, cytotoxic,

TABLE II

Effect of Macrophage Depletion on Humoral and CMI Responses

Cells	Antibody* response		Cell-mediated response	
	SRC	DNP	Cytotoxic‡ % Lysis ± SE	Mitotic§ stimulation index
	AFC/culture ± SE			
CBA spleen	2208 ± 807	515 ± 167	84 ± 3.1	6.2
CBA lymphocytes‖	13 ± 10	450 ± 186	22 ± 2.8	2.8

* Antibody response was measured after 3.7 days. Each value represents the arithmetic mean of three cultures ± the standard error of the mean.

‡ Cytotoxic response at day 6 at a lymphocyte to target cell ratio of 50 to 1 for 200 min or equivalent dilution of cultured lymphocytes. Specific lysis is shown.

§ Stimulation index of the one way mixed lymphocyte reaction at day 4. The actual DPM of the allogeneic spleen cell combination (CBA spleen cells plus mitomycin C-treated BALB/c spleen cells) were 320,000 ± 87,000, the calculated control value 51,000. Purified lymphocytes resulted in the allogeneic combination in 12100 ± 3500 DPM, the calculated control value was 4200 DPM.

‖ Adherence column filtrate of CBA spleen cells by the method of Shortman et al. (21).

mitotic, and antibody producing, were initiated from the same cell pools. The antibody and mitotic responses were assayed at day 4, whereas the cytotoxic response was measured at day 6. Since the survival of unfractionated spleen cells after 6 days' culture was much greater than that of the macrophage-depleted cultures (10–15% to 1–3%), the cytotoxic response was compared on a culture basis rather than on a surviving cell basis, as is routine for measurement of humoral responses (3). Thus, at a ratio of 50 cultured viable cells from cultures of unfractionated CBA spleen cells to 1 ^{51}Cr-labeled target cell, 84% ± 3.2 lysis was obtained, whereas cultured cells derived from macrophage-depleted cell pools assayed at the equivalent dilution resulted only in 24% ± 2.4 lysis. Even if the results were expressed on a surviving cell basis, there was much more lysis obtained with unfractionated spleen cells than with fractionated spleen cells.

To obtain a more quantitative estimate of the effect of macrophage removal of the generation of a cytotoxic immune response, cultured lymphocytes derived from the whole spleen as well as the fractionated cell pools were assayed for cytotoxicity at various ratios of cytotoxic lymphocytes (CL) to target cells. Fig. 1 demonstrates that while the unfractionated spleen cells lysed 20% of the target cells at a ratio of 2 to 1, the purified lymphocytes could only cause this degree of lysis at a "ratio" of 100 to 1. Thus, macrophage depletion caused a 50-fold diminution in cytotoxic activity. This is comparable with the usual degree of depression of the SRC response (Table I; references 3, 5).

The Effect of Anti-Macrophage Serum on the Cytotoxic Response.—Specific

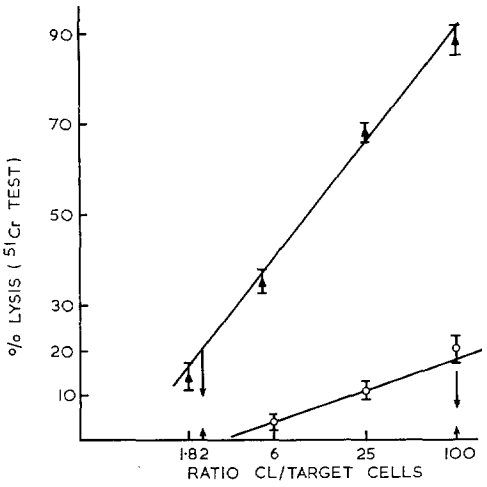

FIG. 1. Effect of macrophage depletion upon the capacity of CBA spleen cells to generate a CMI in vitro. (▲—▲—▲, CBA spleen cells; O—O—O, purified lymphocytes.) All experiments described in figures were assayed at day 6 with equivalent dilutions for spleen-derived and lymphocyte-derived cultured cells. Each point represents the mean ± SD of three determinations. Similar results were obtained in six different experiments.

rabbit anti-mouse macrophage antiserum (AMS) inhibits macrophage-dependent antibody responses, such as the response to SRC (3, 5) or DNP FγG (7) but does not diminish macrophage-independent responses such as those to POL or its conjugates (3, 7). Furthermore it was shown that a combination of adherence column fractionation and AMS provides a more stringent test for macrophage requirements than either method alone (3). Thus, the combined effect of macrophage removal and AMS on the generation of CL was investigated (Fig. 2). There was no residual cytotoxicity demonstrable at day 6 of culture, when macrophage-depleted lymphocytes were cultured together with anti-macrophage serum. The specificity and efficiency of the AMS used was established by control cultures (Materials and Methods, Table I). Thus, the cytotoxic reaction against cellular BALB/c alloantigens

was abrogated by the combined use of specific AMS together with adherence column purification indicating that the response was entirely macrophage dependent.

Reconstitution of the Cytotoxic Response with Peritoneal Exudate Cells (PEC).—Adherence columns retain some lymphocytes as well as phagocytes. For example, antibody-forming cells are selectively removed by these columns (2) as are up to 40–50% of splenic lymphocytes (21). It was thus possible that the diminution of the allograft response caused by the column fractionation procedure might have been due to reasons other than macrophage depletion. Thus experiments were performed to determine whether enriched populations of

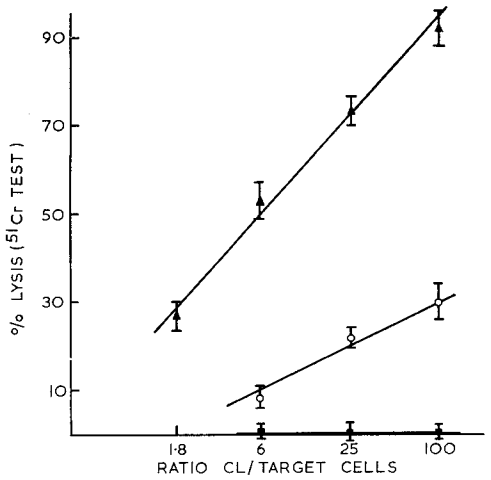

FIG. 2. Effect of the combined use of column purification and specific rabbit anti-macrophage serum upon the capacity of CBA spleen cells to generate a CMI in vitro. (▲—▲—▲, spleen cells; ○—○—○, purified lymphocytes; ■—■—■, purified lymphocytes plus 15% specific AMS during the 6 day culture.) Each point represents the mean ± SD of three determinations.

macrophages could restore the cytotoxic response of lymphocytes. Since PEC populations also contain T lymphocytes, and since the allograft response in vitro is caused by T lymphocytes (29, 14), the PEC were treated with AKR anti-ΘC$_3$H serum and complement before use in culture. Fig. 3 shows that as few as 10^5 anti-Θ-treated CBA PEC significantly enhanced the cytotoxic response of CBA lymphocytes. Optimal restoration required 3×10^6 PEC per culture of 60×10^6 lymphocytes.

Locus of Macrophage Function in the Cytotoxic Response.—In some circumstances macrophages can become activated into cytotoxic effector cells (30, 31). It was thus important to determine whether the diminished cytotoxic response obtained with purified populations of lymphocytes was due to a failure of the induction of the allograft response, or whether macrophages were

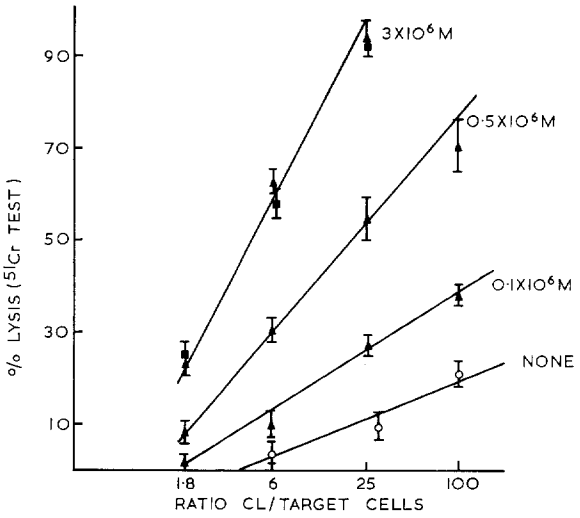

FIG. 3. Reconstitution of the CMI in vitro by peritoneal exudate cells (PEC) depleted of T lymphocytes when added before culture to purified lymphocytes (○—○—○, response of purified lymphocytes). Addition of 3×10^6 PEC (▲—▲—▲) resulted in a complete reconstitution of the CMI to that obtained with the "original" spleen cells (■—■—■). The cytotoxicity assay was performed for all cell populations at the dilution of cultured cells, which resulted in the original spleen cells in the given ratio CL to target cells.

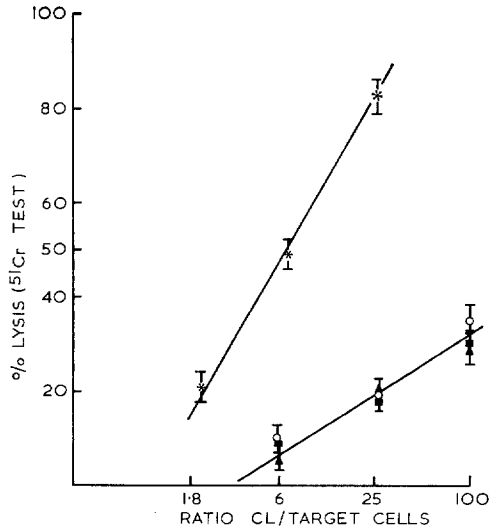

FIG. 4. Effect of peritoneal exudate cells when added after culture to cytotoxic lymphocytes derived from purified lymphocytes. CBA spleen cells (✳—✳—✳); purified lymphocytes (○—○—○); purified lymphocytes plus 5×10^5 PEC (■—■—■); purified lymphocytes plus 1.5×10^6 PEC (▲—▲—▲).

needed for the actual cytotoxic effector phase, as measured by the ^{51}Cr-release test. Thus various numbers of PEC were added to cultured purified lymphocytes just before the ^{51}Cr assay. No enhancement of the cytotoxic activity was found (Fig. 4). In another set of experiments, the cytotoxic activity of unfractionated CBA spleen cells activated in vitro against H-2^d alloantigen was measured in the ^{51}Cr-release test in the presence of 15% AMS. No significant depression of the cytotoxic activity was found (Table III).

TABLE III

Effect of Anti-Macrophage Serum (AMS) upon the Cytotoxic Activity

Serum	% Specific lysis (various ratios lymphocytes to target cells)		
	50/1	10/1	2/1
NRS*	91 ± 1.3	63 ± 1.5	41 ± 2.9
AMS No. 2	94 ± 3.7	62 ± 2.4	40 ± 1.6
AMS No. 6	83 ± 1.4	59 ± 4.1	43 ± 2.3

CBA spleen cells were activated in vitro against H-2^d alloantigens. Cytotoxic activity was determined after 6 days' culture in the ^{51}Cr-release assay in the presence of 15% NRS or 15% anti-macrophage-specific rabbit serum (AMS). Each value represents the arithmetic mean of three determinations ± SEM.

* Normal rabbit serum [NRS was subjected to the same absorption procedure as the AMS (3)].

TABLE IV

Macrophage Dependence of Cytotoxic Response using Subcellular H-2^d Alloantigen

Cells	% Specific lysis (varions ratios lymphocytes to target cells)		
	200/1	40/1	8/1
CBA spleen	14.8 ± 2.0	11.4 ± 2.6	3.08 ± 1.0
Lymphocytes*	0.7 ± 0.95	0	0
Lymphocytes‡ + PEC	9.3 ± 1.8	7.4 ± 0.5	2.9 ± 1.88

All cultures contained the same amount of subcellular H-2^d alloantigen. Cytotoxic assay was performed after 6 days at various ratios for 6 hr. Each value represents the arithmetic mean of three determinations ± SEM. Similar results were obtained in three different experiments.

* Active adherence column filtrate derived from the same pool of CBA spleen cells.

‡ Lymphocytes reconstituted with 2.5×10^6 AKR anti-ΘC3H serum plus complement-treated PEC per culture.

Macrophage Dependence of the Cytotoxic Response to Subcellular Antigens.— The requirement for macrophages of the humoral immune response against whole SRC in vitro can be bypassed by using subcellular or soluble SRC preparations (3). In order to investigate whether the critical function of macrophages during the induction phase of the cytotoxic allograft response is to reduce cell-bound alloantigens to a smaller and more immunogenic form, a subcellular antigenic preparation derived from DBA/2 P-815X-2 tumor cells (H-2^d) was used as antigen.[2] Table IV demonstrates that the spleen cells

respond to this form of antigen, which was unlike the minimal response obtained with purified lymphocytes. The diminished cytotoxic activity of macrophage-depleted lymphocytes could be significantly restored by adding 2.5×10^6 PEC per culture (Table IV). Thus the role of macrophages in the CMI was not solely to reduce antigens to a smaller size.

DISCUSSION

The results as described above provide strong evidence for the absolute requirement of macrophages in a mouse allograft response in vitro. The use of a column purification procedure demonstrated that the cytotoxic response was depressed about 50-fold, to a similar degree as the classical macrophage-dependent SRC response (Table I, Fig. 1). However, since the active adherence column separation of Shortman et al. (21) also traps certain classes of lymphocytes, it was essential to confirm this proposal by macrophage restoration experiments. To avoid the possibility the PEC may contain significant numbers of precursors of cytotoxic lymphocytes, only PEC treated with AKR anti-Θ serum plus complement were used for these experiments. Since T-depleted PEC (85% macrophages) restored the allograft reaction up to normal levels, this confirmed that the majority of the cytotoxic response was macrophage dependent. Cell separation procedures alone did not entirely abrogate the cytotoxic response (Fig. 1), raising the possibility that there may be a minor component of the response that was macrophage independent. Since the combined use of AMS and cell separation procedures provides a more stringent test for macrophage requirements than either method alone (3), experiments of this type were performed. After the combined use of both techniques there was no residual cytotoxicity (Fig. 2), demonstrating that the whole of the response to cellular alloantigens was macrophage dependent. Using an in vitro xenograft system, Lonai and Feldman found, using only cell separation methods, that the cytotoxic response was suppressed, but not abolished (32).

The locus of action of macrophages in the CMI was investigated. Since macrophages are known to be essential for the MLC (Table I, references 11–13) which is considered to be equivalent to the antigen recognition phase of an allograft reaction (11–13), it was possible that macrophages may be essential for the induction phase of the CMI. Alternatively, since macrophages may in some circumstances act as cytotoxic effector cells (30, 31) it was possible that macrophages were the actual killer cells. As both macrophages and activated lymphocytes are trapped by the "adherence column" technique, (21 and Shortman, K., personal communication) two indirect experimental approaches were chosen in order to check the role of macrophages in the cytotoxic effector phase. However, addition of macrophages to cultured purified lymphocytes at day 6 did not result in any cytotoxicity. Moreover, the presence of AMS during the ^{51}Cr-release assay did not influence the cytotoxic response obtained.

Thus we conclude that macrophages were only involved in the induction phase of the cytotoxic response.

The actual role of macrophages in immunological responses is not entirely known. As the humoral immune response to subcellular SRC fractions, unlike the response to whole SRC, is macrophage independent (3), Shortman et al. (2, 5) proposed that macrophages may merely reduce antigens to a smaller and more immunogenic size. This may not be the only functions of macrophages as Feldmann (7) has demonstrated recently that all so far tested humoral responses requiring the cooperation of T and B lymphocytes also required the participation of macrophages. This was the case even with antigens of small size, such as DNP FγG. Since DNP FγG is a much smaller antigen molecule than the macrophage and T-independent DNP POL (7) or soluble SRC antigen (3), macrophages in this system were participating in the process of T-B collaboration.

Using a subcellular H-2^d antigen preparation derived from the P-815X-2 mastocytoma cell line by papain digestion and KCL extraction,[2] the mode of action of macrophages in the allograft response was investigated. Appropriate concentration of the subcellular fraction immunized spleen cells but could not immunize column-purified lymphocytes (Table III). Since this fraction is smaller in size than POL (16), it suggests that macrophages in this reaction may not merely acting by reducing subcellular antigen to a more suitable size for immunization. Rather it suggests that as in the case of T-dependent humoral responses, macrophages may be necessary to provide a surface of repeating antigenic determinants on which T lymphocytes are to be immunized. If this concept of macrophage function in the CMI to subcellular antigen is correct, then two classes of T cells may be collaborating in the allograft response. Evidence for the existence of such a T-T collaboration has already been obtained in the graft-*versus*-host response (33).

SUMMARY

An efficient cell separation procedure and specific anti-macrophage serum were used to investigate the requirement of macrophages in the in vitro allograft response of mouse lymphoid cells. The efficiency of the macrophage-depletion procedure used and the undiminished capacity of the purified lymphocytes to respond were verified by also testing the antibody responses to sheep red cells (SRC) and dinitrophenylated polymeric flagellin (DNP POL) as well as the proliferative response to allogeneic cells. It was found that the generation of cytotoxic lymphocytes were diminished after macrophage depletion by surface adherence. The combination of anti-macrophage serum and column purification resulted in the total abolition of cytotoxic activity. The cell-mediated immune response was restored completely by addition of peritoneal macrophages, with as few as 1 macrophage to 600 lymphocytes permitting a significant restoration. Macrophages were not involved in the cytotoxic effector

phase, but were essential in immune induction. A subcellular H-2 alloantigen preparation was only immunogenic in the presence of macrophages, indicating that a mere reduction in the size of the antigen from cell-bound alloantigens to membrane fragments was not the sole function of macrophages. The results suggest that macrophages collaborate with T cells in the initiation of an allograft response in vitro.

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