

## LYSIS OF INDUCER T CELL CLONES BY ACTIVATED MACROPHAGES AND MACROPHAGE-LIKE CELL LINES\*

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Efficient elimination of foreign substances or organisms is the hallmark of an effective immune response. Most responses are initiated by inducer T cells that corecognize foreign determinants and I region products on antigen-presenting cells (APC)<sup>1</sup> (1–3). A lymphokine produced by inducer cells as a result of this interaction is macrophage-activating factor (MAF) (4–6). MAF activates macrophages to express lytic activity after a second ‘triggering’ signal such as phagocytosis (7–10). Lysis by activated macrophages is an important element of resistance to bacteria, parasites, and tumor cells (11–16).

An equally important component of macrophage-mediated protection is the prevention of excessive immunologic damage to host tissues, once the foreign agent has been eliminated (17). In principle, activated macrophages that destroy infected or cancerous cells might also dampen immune responses by lysing inducer cells that initiate the responses. We have tested this hypothesis using clones of hapten-specific inducer T cells and APC. We find that corecognition of antigen and class II major histocompatibility complex (MHC) products on a subset of APC results in (a) production of MAF (and other lymphokines), (b) triggering of macrophage-mediated cytolysis, and (c) death of the inducer clone. We suggest that this sequence of interactions may limit the duration of specific immune responses by the depletion of inducer T cells that are required to initiate and maintain them.

### Materials and Methods

*Mice.* Female C57B1/6 (B6), BALB/c, A/J, and (BALB/c × A/J)F<sub>1</sub> (CAF<sub>1</sub>) mice were obtained from The Jackson Laboratory, Bar Harbor, ME, and used at 6 wk to 4 mo of age.

*Antigens.* *p*-Arsanilic and *p*-sulphanilic acid (AR, SA) (Eastman Laboratory and Specialty Chemicals, Rochester, NY) were diazotized and conjugated to bovine gamma globulin (BGG) (Sigma Chemical Co., St. Louis, MO) as described (18). Haptenation ratios (moles [AR-TYR + AR-HIS]/mole protein) were estimated using an extinction coefficient

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<sup>1</sup> *Abbreviations used in this paper:* APC, antigen-presenting cells; AR, *p*-azobenzenearsonate; BCG, bacillus Calmette-Guerin; BGG, bovine gamma globulin; Con A, concanavalin A; DME, Dulbecco's modified Eagle's medium; IL-1, IL-2, interleukin 1 and 2; KLH, keyhole limpet hemocyanin; MAF, macrophage-activating factor(s); MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; PMA, phorbol myristate acetate; SA, *p*-azobenzenesulphonate.

of  $8.15 \text{ mM}^{-1}$  in 0.1 N NaOH at the isobestic point of 350 nm (19).

*Monoclonal Antibodies and Antisera.* Cells of the hybridomas 14.4.4s (I-E<sup>d,k,p,r</sup>:Ia.7) (20) and MKD6 (I-A<sup>d,q</sup>:Ia.16) (21 and unpublished results) were cultured in RPMI 1640 with 10% gamma globulin-free horse serum or 10% fetal calf serum. Antibodies were purified from culture supernatants using protein A-Sepharose or DEAE cellulose. Culture supernatant of the hybridoma 10.2.16 (I-A<sup>k</sup>) (22) was a gift from R. Rosenson, Tufts University Medical Center.

*Derivation and Culture of Arsonate-reactive T Cell Clones.* CAF<sub>1</sub> mice suppressed for production of the cross-reactive idiotype for arsonate were a kind gift from Dr. A. Marshak-Rothstein and Dr. M. Geffer. CAF<sub>1</sub> mice were suppressed with a single intraperitoneal injection of monoclonal antiidiotype (23) and immunized with 100  $\mu\text{g}$  of AR-KLH (keyhole limpet hemocyanin) in Freund's complete adjuvant intraperitoneally 18 d later.

Clone Ly1/A5 was derived from singly immunized mice while clone Ly1/A7 was obtained from hyperimmune animals. Immune spleen cells were cultured at a concentration of  $5 \times 10^7$  cells/ml in Dulbecco's modified Eagle's medium (DME) containing 10% selected fetal calf serum and additives (Gibco Laboratories, Grand Island, NY) as described (24) and 50  $\mu\text{g}/\text{ml}$  AR-BGG. Cells surviving after 8 d of culture were recultured at  $2 \times 10^5/\text{well}$  with  $5 \times 10^6$  irradiated (2,000 rad) CAF<sub>1</sub> spleen cells/well in 24-well culture dishes (Linbro Chemical Co., Hamden, CT) in medium containing 50  $\mu\text{g}/\text{ml}$  AR-BGG as above. Arsonate-reactive T cells were maintained by weekly supplementation of all cultures with irradiated CAF<sub>1</sub> spleen cells and antigen. Cultures were tested periodically for the presence of cells proliferating specifically to AR-BGG presented on syngeneic spleen cells. Selected cultures were cloned by limiting dilution in Falcon 3040 microtiter plates (Falcon Labware, Oxnard, CA) containing  $5 \times 10^5$ – $10^6$  irradiated spleen cells and 50  $\mu\text{g}/\text{ml}$  AR-BGG in DME with 10% fetal calf serum, 20% P388D<sub>1</sub> supernatant as a source of interleukin 1 (IL-1) (25, 26) and 2% concanavalin A (Con A)-depleted spleen conditioned medium as a source of IL-2 (27). Clones were tested for specific proliferation to AR-BGG presented on syngeneic spleen cells. All clones selected for expansion recognized the arsonate hapten on several different carrier proteins. Their surface phenotype was Thy-1<sup>+</sup>Ly-1<sup>+</sup>Ly-2<sup>-</sup> using the unlabeled antibody method (27).

*Cell Lines.* The cell lines ORA Ia (28), WEHI-3 (29), P815 (30), P388D<sub>1</sub> (31), and M12.4.1 (32) were grown in RPMI 1640 supplemented with 5% fetal calf serum. The cloned monocyte/macrophage-like cell ORA Ia was obtained from Dr. C. Reinisch, Tufts University Medical Center and the cloned B cell lymphoma M12.4.1 from Dr. L. Glimcher, Massachusetts General Hospital, Boston. ORA Ia, WEHI-3, P388D<sub>1</sub>, and M12.4.1 cells present antigen to clones Ly1/A5 and Ly1/A7, as assayed by specific production of IL-2 (see Figs. 5, 6). P815 cells, although of H-2<sup>d</sup> haplotype, do not express Ia antigens (27) and do not present antigen to the clones. For most experiments requiring macrophage-like APC, the cloned ORA Ia cells were used in preference to WEHI-3 or P388D<sub>1</sub>. Most or all ORA cells are Ia<sup>+</sup> by fluorescence (27), and 80–100% are killed by monoclonal antibodies to I-A<sup>d</sup> or I-E<sup>d</sup> plus complement, in contrast to only 10–30% of the WEHI-3 and P388D<sub>1</sub> sublines maintained in this laboratory. Levels of Ia expression on ORA cells are stable over time; on WEHI-3 and P388D<sub>1</sub> Ia expression is normally low, but can be induced by incubation with T cell supernatants (unpublished results).

*Proliferation Assays.* The response of T cell clones to antigen and APC was measured as [<sup>3</sup>H]thymidine uptake ("proliferation") (27).  $5 \times 10^5$  irradiated spleen cells (2,000 rad) or  $10^4$  irradiated (13,000 rad) or mitomycin C-treated (50  $\mu\text{g}/\text{ml}$ , 30 min, 37°C) tumor cells were incubated in microtiter wells with  $2$ – $5 \times 10^4$  T cells and antigen in 0.2 ml DME with 5% fetal calf serum, 2 mM L-glutamine, 50  $\mu\text{M}$  2-mercaptoethanol, and 1 mM Hepes (assay medium). Cells were pulsed at 20–25 h with 1  $\mu\text{Ci}$  [<sup>3</sup>H]thymidine (New England Nuclear, Boston, MA) and harvested at 35–40 h. Results are plotted as the mean [<sup>3</sup>H]-thymidine uptake in duplicate or triplicate wells.

*Production of Lymphokine-containing Supernatants.*  $5 \times 10^4$  cloned T cells were incubated in 0.2 ml assay medium with (a) 100  $\mu\text{g}/\text{ml}$  AR-BGG and  $10^6$  irradiated CAF<sub>1</sub> spleen cells (or adherent cells derived from them); (b) 100  $\mu\text{g}/\text{ml}$  AR-BGG and  $10^5$  irradiated or mitomycin C-treated ORA cells; or (c) 2  $\mu\text{g}/\text{ml}$  Con A. Supernatants were harvested at 15

h; their content of IL-2 (and of MAF) was approximately equal.

*Lymphokine Assays.* IL-2 was assayed, as previously described, by its ability to support proliferation of an IL-2-dependent T cell clone (27). MAF was assayed as described (6); briefly, T cell supernatants were incubated in duplicate wells with  $3 \times 10^4$  ORA cells or with adherent cells derived from  $2 \times 10^5$  resident (unelicited) peritoneal macrophages from CAF<sub>1</sub> or B6 mice. After incubation for 20 h to allow macrophage activation,  $3 \times 10^4$  <sup>51</sup>Cr-labeled P815 cells were added together with  $5 \times 10^{-8}$  M phorbol myristate acetate (PMA) as a trigger for cytolysis. <sup>51</sup>Cr released into the supernatant was measured 20 h after the addition of target cells. The percent specific lysis was calculated from the mean <sup>51</sup>Cr release in duplicate or triplicate wells, as [(release in experimental wells – spontaneous release) × 100]/(maximal release – spontaneous release) (26). Maximal release was measured after treatment with 2% Nonidet P-40 or 3N HCl.

*Bystander Cell Lysis.* In a variant of the MAF assay, T cell clones were examined for their ability to both activate and trigger cytolysis.  $5 \times 10^4$  cloned T cells were incubated with  $3 \times 10^4$  <sup>51</sup>Cr-P815 cells, antigen, and APC [(a) adherent cells from  $2 \times 10^5$  resident peritoneal macrophages; (b)  $10^5$  irradiated or mitomycin C-treated ORA or M12.4.1 cells; (c)  $10^6$  bacillus Calmette-Guerin (BCG)-activated peritoneal cells obtained as described (7); or (d) ORA cells or peritoneal adherent cells activated with T cell supernatants as described above]. In some experiments, the APC were pulsed with antigen (37°C, 1 h) and excess free antigen was removed by washing. <sup>51</sup>Cr released into the supernatant was measured at 20 h for nonactivated cells, and at 4–6 h for cells activated with BCG in vivo or with T cell supernatants in vitro. Percent specific lysis was calculated as described above.

*Estimation of Surviving T Cells After Culture with ORA Cells and Antigen.*  $3\text{--}5 \times 10^4$  cloned T cells,  $3 \times 10^4$  irradiated or mitomycin C-treated ORA cells, and the indicated concentrations of antigen were incubated in 0.2 ml of assay medium in round-bottomed microtiter wells. Excess antigen was removed by washing after 1–16 h. ORA cells pulsed with antigen (37°C, 1 h) were equally effective. 2–3 d later,  $5 \times 10^5$  irradiated CAF<sub>1</sub> spleen cells and 30–50 µg/ml AR-BGG were added to all wells. The wells were pulsed with [<sup>3</sup>H]thymidine and harvested as for a standard proliferation assay. [<sup>3</sup>H]thymidine incorporation was taken as an estimate of the number of surviving T cells.

## Results

*Characteristics and Specificity of T Cell Clones.* Arsonate-specific inducer T cell clones were derived from CAF<sub>1</sub> mice immunized with AR-KLH. Clones Ly1/A5 and Ly1/A7, used for these experiments, recognize AR conjugates of several different carrier proteins but not the structurally similar SA conjugates (Fig. 1A). Both clones also recognize I-A<sup>d</sup> (Fig. 1B). Upon stimulation, the clones rapidly release a variety of lymphokines (these promote growth of T cells, mast cells, and B cells, cause differentiation of thymocytes into cytolytic cells, and increase Ia expression and activity of APC (manuscript in preparation)). In this report we focus on the biologic effects of MAF.

*Production of MAF by Inducer T Cell Clones.* After stimulation with Con A or antigen-pulsed APC, clones Ly1/A5 and Ly1/A7 produce MAF that activate peritoneal adherent cells to lyse <sup>51</sup>Cr-labeled tumor cells (Fig. 2). Active supernatants (produced by incubation of the clones with Con A or antigen-pulsed APC) were added to cultures of resident peritoneal adherent cells, and 24 h later <sup>51</sup>Cr-labeled P815 target cells were added together with PMA as a trigger for cytolysis. Fig. 2 shows that T cell supernatants contain MAF activity, since incubation of peritoneal cells with increasing amounts of supernatant results in increasing lysis of P815 target cells in the presence of the PMA trigger. Incubation with PMA alone or supernatant alone does not cause detectable lysis (Fig. 2), showing that both steps, activation and triggering, are necessary for lysis of

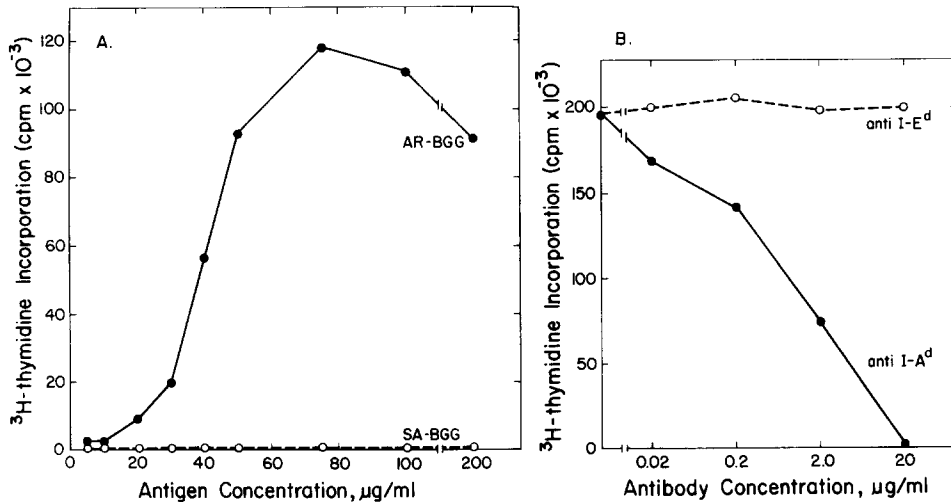


FIGURE 1. Antigen and MHC specificity of Cl.Ly1/A5. (A)  $5 \times 10^4$  Cl.Ly1/A5 cells were incubated with  $5 \times 10^5$  irradiated CAF<sub>1</sub> spleen cells and the indicated concentrations of antigen. (B) Cultures contained  $5 \times 10^4$  Cl.Ly1/A5,  $5 \times 10^5$  irradiated CAF<sub>1</sub> spleen cells, 100 μg/ml AR-BGG, and the purified monoclonal antibodies MKD6 (anti-I-A<sup>d</sup>) and 14.4.4 (anti-I-E<sup>d</sup>). [<sup>3</sup>H]thymidine incorporation was measured between 24 and 40 h.

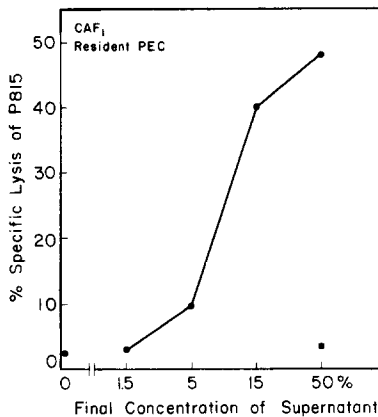


FIGURE 2. Production of MAF by Cl.Ly1/A5 in response to Con A. Supernatants were obtained from  $5 \times 10^4$  Cl.Ly1/A5 cells incubated with 2 μg/ml Con A in 0.2 ml medium for 15 h (see Materials and Methods). Adherent cells derived from  $2 \times 10^5$  resident CAF<sub>1</sub> peritoneal macrophages were incubated with the indicated concentrations of supernatant for 24 h.  $3 \times 10^4$  <sup>51</sup>Cr-labeled P815 cells were then added together with  $5 \times 10^{-8}$  M PMA to trigger cytotoxicity. <sup>51</sup>Cr released into the supernatant was measured 20 h later. (●) PMA, (■) no triggering agent. Spontaneous lysis of P815 was 28%. Similar results were obtained with supernatants from Cl.Ly1/A7 or with supernatants obtained after stimulation of the clones with antigen and APC.

P815 cells by peritoneal macrophages.

Inducer T cells produce MAF only after precise corecognition of antigen and I-A, or after stimulation by Con A. However, once produced, MAF activates macrophages from mice of various H-2 haplotypes (4, 6; not shown).

*Triggering of Activated Macrophages by Inducer T Cells.* MAF produced by T

cells activates macrophages but does not trigger cytolysis (Fig. 2). In contrast, T cells themselves can trigger lytic activity if they corecognize antigen and I-A on the surface of activated macrophages (Fig. 3, Table I). Activated peritoneal macrophages expressing I-A<sup>d</sup>, which were lytic for P815 cells in the presence of PMA, were obtained from BCG-injected CAF<sub>1</sub> mice. The T cell clones Ly1/A5 and Ly1/A7 can rapidly trigger these cells to lyse P815 target cells in the presence of AR-BGG, but not in the absence of antigen or in the presence of the nonstimulatory antigen, SA-BGG (Fig. 3). AR-BGG in the absence of T cells does not stimulate cytolysis. Lysis is dependent on the presence of peritoneal cells; T cells plus AR-BGG alone do not mediate lysis (Fig. 3).

In addition to recognition of antigen, lysis required corecognition by the inducer T cells of the correct I region product on the surface of the activated macrophages (Table I). Resident peritoneal macrophages from BALB/c and A/J mice were activated by incubation with T cell supernatants. They were then triggered to lyse <sup>51</sup>Cr-labeled P815 target cells by the addition of clone Ly1/A5 and AR-BGG, or PMA. Table I shows that PMA can trigger lysis by activated peritoneal adherent cells from both BALB/c and A/J mice, while Cl.Ly1/A5 triggers lysis only by activated BALB/c cells, which, unlike A/J cells, express the stimulatory protein I-A<sup>d</sup> (Table I). Lysis triggered by PMA is similar in magnitude to that triggered by the T cell clone and antigen (Table I). Nonactivated BALB/c cells do not mediate lysis (not shown).

These findings demonstrate that cytolysis is triggered by inducer T cells after specific corecognition of antigen and class II molecules on activated peritoneal cells. Since production of MAF is also stimulated by corecognition (Fig. 2), we found, as expected, that Cl.Ly1/A5 would sequentially activate and trigger I-A<sup>d</sup>-bearing peritoneal adherent cells after 16–20 h of in vitro incubation (measured by lysis of bystander P815 cells). Again lysis depended on incubation of the clone with AR-BGG and adherent cells bearing I-A<sup>d</sup> (not shown). Use of the cloned B cell line M12.4.1 to present antigen showed that the T cell clone did not itself

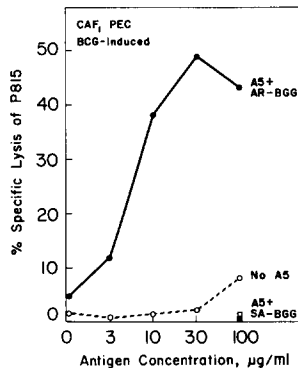


FIGURE 3. Cl.Ly1/A5 can trigger cytolysis by BCG-activated H-2<sup>d</sup> peritoneal macrophages in the presence of appropriate antigen. 10<sup>6</sup> peritoneal cells from CAF<sub>1</sub> mice injected with BCG were incubated with 7 × 10<sup>4</sup> Cl.Ly1/A5, 3 × 10<sup>4</sup> <sup>51</sup>Cr-P815 cells, and antigen. <sup>51</sup>Cr released into the supernatant was measured in duplicate wells 4 h later. (●) Cl.Ly1/A5 + AR-BGG; (□) Cl.Ly1/A5 + SA-BGG; (○) AR-BGG, no Cl.Ly1/A5; (■) Cl.Ly1/A5 + AR-BGG, no peritoneal cells. Spontaneous lysis of P815 was 11%.

TABLE I  
*Lysis of Bystander P815 Cells by Activated and Triggered Peritoneal Adherent Cells*

Activated peritoneal cells	Trigger	Percent specific lysis of P815
—	—	0
—	PMA	1
—	Cl.Ly1/A5 + AR-BGG	1
BALB/c	—	2
BALB/c	PMA	37
BALB/c	Cl.Ly1/A5	2
BALB/c	AR-BGG	5
BALB/c	Cl.Ly1/A5 + AR-BGG	49
A/J	—	0
A/J	PMA	35
A/J	Cl.Ly1/A5	0
A/J	AR-BGG	3
A/J	Cl.Ly1/A5 + AR-BGG	5

Adherent cells derived from  $2 \times 10^5$  resting peritoneal cells from BALB/c or A/J mice were activated by incubation in 0.1 ml with 50% MAF-containing supernatants from Cl.Ly1/A5 stimulated with 2  $\mu\text{g/ml}$  Con A. 24 h later,  $3 \times 10^4$   $^{51}\text{Cr}$ -labeled P815 cells were added together with  $5 \times 10^{-8}$  M PMA or  $3 \times 10^4$  Cl.Ly1/A5 cells and 50  $\mu\text{g/ml}$  AR-BGG as indicated.  $^{51}\text{Cr}$ -release was measured 6 h later in triplicate wells. Spontaneous release was 9%.

mediate lysis of P815 cells after stimulation (see Use of Cloned APC Lines).

*Lysis of Inducer T Cells by Activated Macrophages.* We then asked how the development of cytolytic activity by adherent cells affected activation and subsequent proliferation of the inducer T cell itself. Increasing numbers of Cl.Ly1/A5 cells were added to antigen-pulsed adherent cells from the spleen or peritoneal cavity (the clones are normally expanded on irradiated splenic adherent cells). We measured activation of the T cell as (a) IL-2 production, which occurs within the first 16 h, and is a direct measure of the number of activated T cells (33); (b) DNA synthesis, i.e., incorporation of [ $^3\text{H}$ ]thymidine between 24 and 40 h; and (c) numbers of viable T cells after 50 h in culture.

T cells incubated with peritoneal adherent cells incorporated about 10-fold less [ $^3\text{H}$ ]thymidine in response to antigen than T cells incubated with splenic adherent cells (Fig. 4B). Since decreased DNA synthesis can reflect noncytolytic inhibitory processes, we also measured T cell viability by trypan blue exclusion. Numbers of viable T cells decreased by  $\sim 50\%$  after incubation with antigen-pulsed peritoneal adherent cells, but increased as expected after incubation with antigen-pulsed splenic cells (Table II). These effects cannot be ascribed to lack of activation by peritoneal APC, since both spleen and peritoneal adherent cells stimulated production of equivalent amounts of IL-2 in the first 18 h (Fig. 4A, Table II). Control experiments show that spleen and peritoneal cells neither

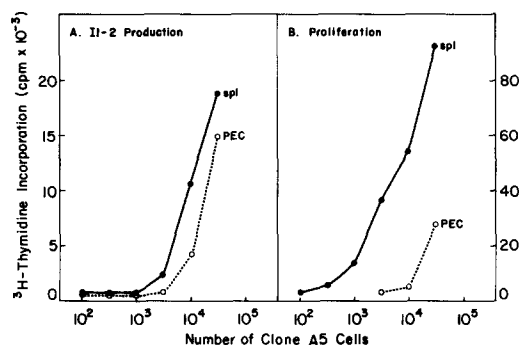


FIGURE 4. Stimulation of Cl.Ly1/A5 by peritoneal and splenic APC. 10<sup>6</sup> irradiated CAF<sub>1</sub> spleen cells or 3 × 10<sup>5</sup> irradiated CAF<sub>1</sub> peritoneal cells were pulsed (37°C, 1 h) with 50 µg/ml AR-BGG. Nonadherent cells and excess antigen were removed by washing, and graded numbers of Cl.Ly1/A5 were added to the wells. (A) Supernatants were removed at 15 h for assay of IL-2 production; (B) [<sup>3</sup>H]thymidine uptake (proliferation) was measured between 20 and 40 h. IL-2 in the supernatants was measured as [<sup>3</sup>H]thymidine uptake by an IL-2-dependent Ly2<sup>+</sup> T cell clone, exposed to a 20% final concentration of the supernatants (27).

TABLE II  
Decrease in Viable Cl.Ly1/A7 Cells After Activation on Peritoneal as Compared with Splenic Antigen-pulsed Adherent Cells

Adherent cells	IL-2 production U/ml	Number of viable T cells
Splenic	0.33	1.87 × 10 <sup>6</sup> (1.76–1.98)
Peritoneal	0.38	0.56 × 10 <sup>6</sup> (0.44–0.68)

10<sup>7</sup> spleen cells or 10<sup>6</sup> resting peritoneal cells (2,000 rad) were pulsed with 50 µg/ml AR-BGG (37°C, 1 h). Excess antigen and nonadherent cells were removed by washing. 10<sup>6</sup> Cl.Ly1/A7 cells were added in 1 ml medium. Samples of supernatant were removed at 18 h and assayed for IL-2 content (27), using spleen Con A-conditioned medium as a standard (1 U/ml). At 50 h the number of viable Cl.Ly1/A7 cells was estimated by trypan blue exclusion. The values given are the mean of duplicate wells (range in parentheses). Control experiments showed that antigen-pulsed splenic and peritoneal adherent cells did not produce IL-2 in the absence of T cells (<0.02 U/ml in 18 h) and that they did not absorb appreciable amounts of IL-2 from culture media containing supernatant from Con A-stimulated Cl.Ly1/A5 cells (0.05 and 0.02 U/ml absorbed by splenic and peritoneal adherent cells, respectively, in 18 h at 37°C). The results are representative of three similar experiments.

produce IL-2 in the absence of T cells, nor absorb IL-2, even if activated by MAF present in Cl.Ly1/A5 supernatant (Table II). The release of IL-2 after incubation with peritoneal adherent cells cannot be attributed to release from dying T cells alone, since inducer T cells produce 20- to 100-fold more IL-2 after specific immunological stimulation than they release after lysis by cytolytic cells (27). In sum, although IL-2 production is similar in both cases, activation by splenic adherent cells results in clonal expansion while activation by peritoneal adherent cells results in depletion of inducer T cells.

*Use of Cloned APC Lines.* Does the same subset of adherent cells that can activate T cell clones also mediate their lysis? One approach to this question was

to compare the cloned APC ORA Ia and M12.4.1, which resemble macrophages and B cells, respectively (28, 32).

Although both ORA and M12.4.1 cells can activate Cl.Ly1/A5 to produce IL-2 (Figs. 5 and 6), ORA cells develop cytolytic activity whereas M12.4.1 cells do not (Fig. 7B). Lysis by ORA cells depends on the presence of Cl.Ly1/A5 and AR-BGG, but does not occur in cultures containing Cl.Ly1/A5 and SA-BGG (Fig. 7A). As in the case of peritoneal macrophages, lysis by ORA cells was divisible into two steps: activation and triggering (not shown). Thus, we have shown that (a) a single I-A<sup>+</sup> cell type (exemplified by the cloned monocyte/macrophage-like ORA cells) can initially activate inducer cells via corecognition of antigen, and later develop cytolytic capacity; (b) development of cytotoxicity requires not only activation of the inducer T cell but also presence of the appropriate APC (ORA vs. M12.4.1).

We measured viability of inducer T cell clones in cultures containing ORA

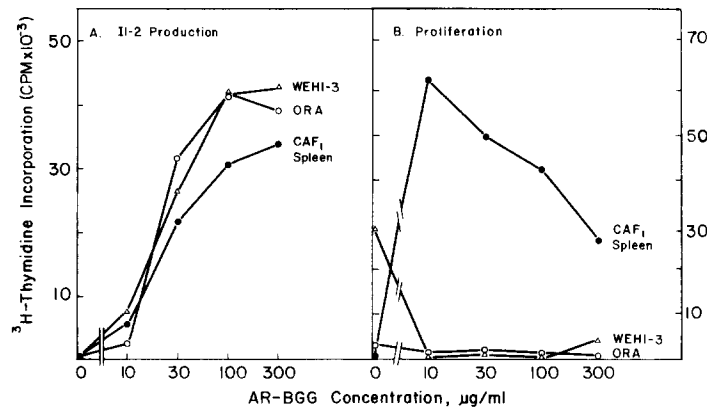


FIGURE 5. Death of Cl.Ly1/A5 after activation with macrophage-like APC and antigen.  $5 \times 10^4$  Cl.Ly1/A5 cells were incubated with  $10^4$  irradiated ORA cells,  $3 \times 10^4$  irradiated WEHI-3 cells, or  $5 \times 10^5$  irradiated CAF<sub>1</sub> spleen cells and antigen. IL-2 production (A) and DNA synthesis (B) were measured as described in the legend to Fig. 4.

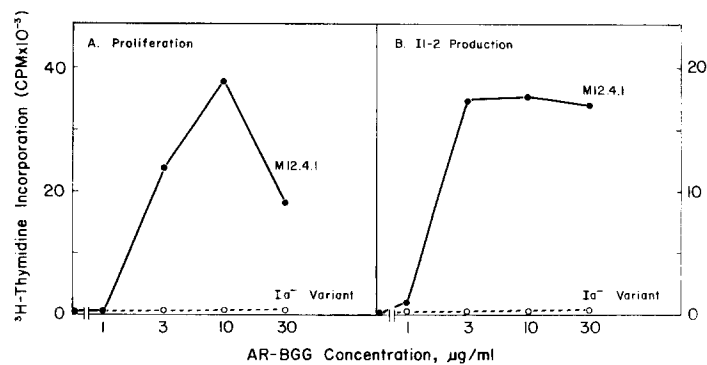


FIGURE 6. Stimulation of Cl.Ly1/A5 by the cloned B lymphoma M12.4.1 and antigen.  $5 \times 10^4$  Cl.Ly1/A5 cells were incubated with antigen and  $10^4$  mitomycin C-treated M12.4.1 cells or an Ia<sup>-</sup> variant derived from it (30). IL-2 production (B) and DNA synthesis (A) were measured as described in Fig. 4.



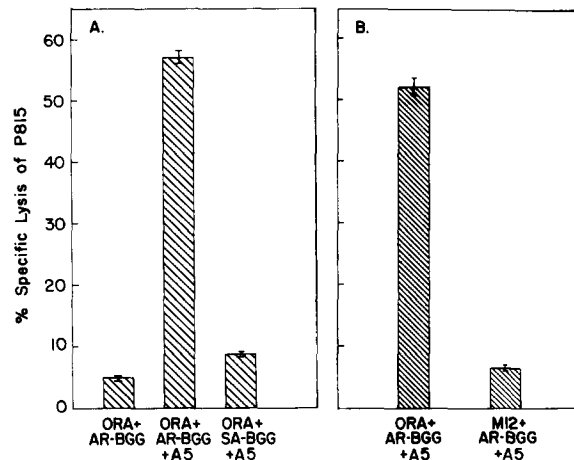


FIGURE 7. Lysis of bystander P815 cells by activated and triggered cloned APC. (A)  $3 \times 10^4$  mitomycin C-treated ORA cells pulsed ( $37^\circ\text{C}$ , 1 h) with  $300 \mu\text{g/ml}$  AR-BGG or SA-BGG were incubated with or without  $3 \times 10^4$  Cl.Ly1/A5 cells and  $3 \times 10^4$   $^{51}\text{Cr}$ -P815 cells for 20 h. (B)  $3 \times 10^4$  mitomycin C-treated ORA cells or M12 cells were incubated with  $3 \times 10^4$  Cl.Ly1/A5 cells,  $100 \mu\text{g/ml}$  AR-BGG, and  $3 \times 10^4$   $^{51}\text{Cr}$ -P815 cells for 20 h. Release is plotted as mean specific lysis  $\pm$  SEM, in triplicate wells. Spontaneous lysis of P815 was 30%.

TABLE III  
*Death of Cl.Ly1/A5 in Cultures Containing ORA Cells and Antigen*

Preincubation	Number of viable T cells	Percent initial number
AR-BGG	$5.2 \times 10^5$	87
ORA	$3.9 \times 10^5$	65
AR-BGG + ORA	No live cells	0
SA-BGG + ORA	$4.2 \times 10^5$	70

$6 \times 10^5$  Cl.Ly1/A5 T cells were incubated with  $3.6 \times 10^5$  irradiated (13,000 rad) ORA cells and  $300 \mu\text{g/ml}$  AR-BGG or SA-BGG as indicated. 18 h later the number of viable T cells in the cultures was estimated by trypan blue exclusion. The results are representative of four similar experiments.

cells and antigen. We measured T cell viability by trypan blue exclusion rather than by  $^{51}\text{Cr}$  release, to avoid problems of quantitation caused by high background release and reabsorption of radiolabel. ORA cells were more efficient than peritoneal cells at causing T cell death: no viable T cells remained after 18 h of incubation with ORA cells pulsed with AR-BGG (Table III). Incubation of Cl.Ly1/A5 with AR-BGG alone, ORA cells alone, or ORA cells pulsed with SA-BGG did not cause appreciable T cell death. The small decrease in viable T cell numbers in the latter groups is due to incubation for 18 h in assay medium lacking IL-2 (Table III).

Death of the T cell clone is a direct consequence of corecognition of antigen and I-A on ORA cells (Fig. 8). The number of viable T cells remaining after initial interaction with irradiated ORA cells was measured as [ $^3\text{H}$ ]thymidine incorporation by the T cells in response to subsequent stimulation by splenic APC and antigen. Incubation of Cl.Ly1/A5 with AR-BGG alone, ORA cells

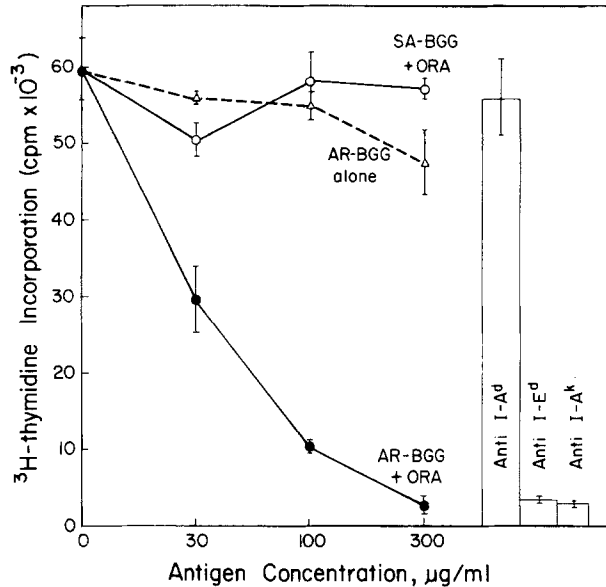


FIGURE 8. Death of Cl.Ly1/A5 in cultures containing ORA cells and antigen.  $3 \times 10^4$  Cl.Ly1/A5 cells were incubated with  $3 \times 10^4$  irradiated ORA cells and antigen as indicated, in the presence or absence of monoclonal antibodies to I-A<sup>d</sup> (MKD6), I-E<sup>d</sup> (14.4.4), and I-A<sup>k</sup> (10.2.16). Antibodies were used at 20 µg/ml with 300 µg/ml AR-BGG. After 16 h of incubation, antibodies and excess antigens were removed by washing. At 48 h,  $5 \times 10^5$  irradiated CAF<sub>1</sub> spleen cells and 50 µg/ml AR-BGG were added to all cultures and [<sup>3</sup>H]-thymidine incorporation was measured between 20 and 40 h later.

alone, ORA cells and SA-BGG, or splenic adherent cells and AR-BGG did not result in loss of subsequent responsiveness to antigen and spleen cells. Incubation with both ORA cells and AR-BGG caused a dose-dependent decrease in the subsequent response (Fig. 8). Inclusion of monoclonal antibodies to I-A<sup>d</sup>, but not to I-E<sup>d</sup> or I-A<sup>k</sup>, during the initial incubation with ORA cells and antigen allowed Cl.Ly1/A5 to respond vigorously to the second stimulus (Fig. 8). Taken together, these results show that corecognition of antigen and I-A on ORA APC leads to lysis of the inducer T cell clone.

Like ORA cells, two other H-2<sup>d</sup> monocyte/macrophage-like cell lines, WEHI-3 and P388D<sub>1</sub>, also caused rapid T cell death in the presence of antigen (not shown). This effect was also apparent when IL-2 production was compared with [<sup>3</sup>H]thymidine incorporation (Fig. 5). IL-2 production is a rapid result of corecognition: peak levels of IL-2 are produced by 12–16 h, while [<sup>3</sup>H]thymidine incorporation occurs between 20 and 40 h, after significant loss in viability of T cells has already occurred (Table III). Cl.Ly1/A5 was incubated with antigen and either syngeneic spleen cells or the monocyte/macrophage lines WEHI-3, P388D<sub>1</sub>, and ORA Ia. Levels of IL-2 production were approximately equal in all cases (Fig. 5A), but T cells incorporated [<sup>3</sup>H]thymidine only in cultures containing splenic APC (Fig. 5B).

### Discussion

Certain types of APC, when activated by MAF produced by inducer T cells, can be triggered to lyse neighboring cells (4–10; Figs. 2, 3, and 7; Table I). This sequence of events plays a central role in resistance to parasites, bacteria, viruses, and tumor cells (3–16). However, normal host cells at sites of inflammation are also vulnerable to damage by activated macrophages (17). We have used MAF-producing inducer T cell clones to determine whether interactions between T cells and APC, in addition to enhancing immune reactions, may also act to curtail its damaging side effects.

Our studies show that inducer T cell clones which corecognize the arsonate hapten and I region products release MAF shortly after stimulation, and can trigger a subset of APC (sensitized by MAF) to express cytolytic activity (Figs. 2, 3, and 7, Table I). This interaction results in lysis of the antigen-specific inducer clone itself (Tables II and III, Fig. 8). Corecognition (the normal stimulus for clonal expansion) is required for both steps in this sequence, the initial production of MAF (4–6; not shown) and subsequent triggering of macrophage cytotoxicity (Figs. 3 and 7, Table I). The most significant aspect of this mechanism is that the initial cell-cell interaction necessary for maximal lysis of inducer T cell clones (Table III, Fig. 8) is precisely the same as for optimal activation, i.e., incubation with I-A<sup>d</sup>-bearing APC and AR-BGG, but not the nonstimulatory analogue SA-BGG (Fig. 1). T cell lysis may preferentially occur as a result of cell-cell contact (8): (a) lysis cannot be mediated by supernatants from activated T cells (Fig. 2) or activated macrophages (not shown); (b) the specific inducer clone (which interacts with activated macrophages via corecognition) is more efficiently lysed than 'bystander' P815 cells (which are at least as susceptible to macrophage-mediated lysis [34]; compare Table III and Figs. 5 and 8 with Fig. 7; Fig. 4 with Figs. 2 and 3). The physiological consequences of these interactions are decreased duration and intensity of macrophage-mediated lysis, which depends on the continued presence of inducer T cells (10), and selective depletion of clones of T cells which corecognize foreign determinants and thus activate macrophage-mediated immunity.

Studies of immunological memory suggest that this mechanism may operate in vivo. Infection of mice with *Listeria monocytogenes* produces activated macrophages that are highly cytolytic and eliminate infecting organisms (4, 11, 13–15). However, persistence of immunity after primary infection with *Listeria* is weak and variable. High levels of adoptive immunity can be transferred by T lymphocytes obtained 6 d after primary infection, but not by lymphocytes obtained on the 10th and subsequent days (11, 13–15).

Morphological studies on the immune response in lymph nodes suggest that this mechanism regulates antimicrobial reactions in vivo (35). Strong lymphoproliferative responses are seen in draining lymph nodes after footpad injections of attenuated paratyphoid organisms (35). This is followed by a rapid increase in dead lymphocytes and phagocytic macrophages that is particularly evident in germinal centers and T-dependent areas of the node (35).

The immune response to bacteria and parasites requires the presence of phagocytic macrophages, since phagocytosis and subsequent "processing" is required for efficient presentation of bacterial antigens (36). Hence, the mechanism

described here may be particularly important in regulating damage to host tissues during microbial infections by activated and phagocytic macrophages. In contrast, activated macrophages are not a major component of the response to viral or protein antigens, perhaps because such antigens are preferentially presented to inducer cells by nonphagocytic APC (dendritic cells; 37). In these cases, inducer T cells may be inactivated by other means, such as production of Ly-2<sup>+</sup> T suppressor cells (38), as well as a nonlytic interaction with APC resulting in loss of inducer function (39).

Our experiments with I-A<sup>+</sup> cell lines suggest that, while certain cells (such as the B cell-like M12.4.1) activate lymphokine production and expansion of inducer T cell clones (Figs. 6 and 7), others (such as the macrophage-like ORA Ia) specifically activate and deplete these clones (Figs. 5 and 8, Table III). Walker et al. (45) and McKean et al. (46) have also observed that I-A<sup>+</sup> macrophage lines are less effective than B cell lines at stimulating DNA synthesis by antigen-specific lymph node T cells and long-term T cell lines, although they can specifically stimulate T cell hybridomas to produce IL-2 (48, 49). In these experiments, macrophage-like APC stimulated heterogeneous T cell populations to incorporate [<sup>3</sup>H]thymidine (45). Our analysis suggests that this may reflect initial production of lymphokines by antigen-specific cells (48, 49; Fig. 5), and subsequent activation of other cells in the culture (47). An important conclusion is that IL-2 production is a more reliable measure than DNA synthesis of the activation of T cell clones by macrophage-like APC; however, DNA synthesis is preferable as an index of clonal expansion.

Like I-A<sup>+</sup> cell lines, I-A<sup>+</sup> nonneoplastic cells purified from lymphoid tissues may also differ in their ability to activate and expand inducer T cells. Steinman et al. (40) and Nussenzweig et al. (41) have shown that macrophages from spleen and peritoneum do not initiate a primary immune response, even though they may display high levels of I-A after incubation with T cell lymphokines. In contrast, nonphagocytic I-A<sup>+</sup> splenic dendritic cells stimulate [<sup>3</sup>H]thymidine incorporation in a primary mixed lymphocyte culture and provide accessory function for the generation of TNP-specific cytolytic T cells (40–43). Another study shows that APC from spleen and lymph nodes, but not peritoneal adherent cells or bone marrow macrophages, stimulate [<sup>3</sup>H]thymidine incorporation by long-term T cell lines reactive to myelin basic protein (44).

We have not examined cloned “dendritic” cell lines such as those derived from the P388 tumor (50). One type of clone derived from the heterogeneous P388 cell line stimulates allogenic and syngeneic mixed lymphocyte reactions (MLR), and has morphological and functional characteristics of dendritic cells (50); a second type expresses equivalent amounts of I-A but does not stimulate in a primary MLR (50). These clones should be examined for sensitization by MAF and expression of lytic activity triggered by inducer cells.

Lysis of inducer clones by macrophage-like cell lines occurred rapidly after corecognition (Table III, Fig. 5). In contrast, analysis of peritoneal adherent cells suggested a slower “cytostatic” effect (10, 17). Although [<sup>3</sup>H]thymidine incorporation between 24 and 40 h was diminished by 90% compared with cultures supplemented with splenic adherent cells (Fig. 4), T cell viability decreased more

slowly, to 50% in 50 h (Table II). Activated peritoneal macrophages have similar effects on tumor cells: DNA synthesis is rapidly inhibited while cell death occurs much later (10, 17). We have not determined the possible mechanisms of cytostasis or lysis (7-9, 12, 16) in our experiments. Lipoxygenase and cyclooxygenase inhibitors (BW755C, 100  $\mu$ M, and indomethacin, 100  $\mu$ M) have no effect, suggesting that arachidonic acid metabolites are not involved.

Splenic adherent cells, which contain both macrophages and dendritic cells (37), permit T cell expansion. This may be because the APC subset permitting T cell proliferation is dominant in mixed cultures. We have found that mixtures of splenic adherent cells and ORA cells (containing numbers of ORA cells that alone would have lysed all T cells) activate T cell clones as efficiently as splenic adherent cells alone (measured by [ $^3$ H]thymidine incorporation; unpublished results).

A second possibility raised by these studies is that splenic macrophages, unlike peritoneal macrophages, do not mediate cell lysis. Macrophages from different tissues, or at different stages of development, are differentially sensitive to activation by lymphokines (10, 17). Once present, the ability to respond to MAF and mediate lysis may be a stable property. This is indicated both by studies of cell lines (6 and this paper) and of differentiated resident macrophages from various tissues (10). We are currently comparing peritoneal macrophages, splenic macrophages, and splenic dendritic cells for cytolytic function after exposure to MAF.

### Summary

We describe a sequence of reciprocal interactions between cloned inducer T cells and antigen-presenting cells (APC) that results in selective depletion of the antigen-reactive inducer cells. We show that corecognition of antigen and I-A by hapten-reactive inducer T cell clones results in (a) release of macrophage-activating factor (MAF) and other lymphokines, (b) expression of lytic activity by a subset of MAF-sensitized APC after triggering, and (3) lysis (mediated by the activated and triggered macrophage) of the inducer T cell clone and other cells in the vicinity. We suggest that this sequence of steps may limit the extent of macrophage-mediated tissue destruction by depleting the specific inducer T cell clones that initiate the response.

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