In Vitro Nonimmunologic Destruction of Cells with Abnormal Growth Characteristics by Adjuvant Activated Macrophages[1] (36295)

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Recently we reported that mice chronically infected with the intracellular protozoa *Toxoplasma gondii* and *Besnoitia jellisoni* have increased resistance to autochthonous and transplanted tumors (1, 2). Studies performed to determine the mechanism(s) of this increased resistance revealed that activated macrophages, but not lymphocytes, from mice chronically infected with the protozoa and the facultative intracellular bacteria *Listeria monocytogenes* were cytotoxic to target cells with abnormal growth characteristics[3] *in vitro* (3). The efferent limb of the activated macrophage mediated *in vitro* cytotoxic reactions appears to be nonimmunologic but discriminatory insofar as only target cells with abnormal growth characteristics were selectively destroyed (3). Target cell destruction was effected by a nonphagocytic mechanism and early degenerative changes were evident by 2 hr after contact with activated macrophages (3).

In separate studies we have shown that primary vaccination with "killed" vaccines (*e.g.*, complete Freund's adjuvant) may be as effective as live chronic infection in inducing long-term protection against antigenically unrelated intracellular infectious challenge, if the inciting antigen is not allowed to dissipate (8). In the companion paper, such vaccination also protected against autochthonous and transplanted tumors in mice (9). The purpose of this report is to describe the cytotoxic effect of macrophages activated by complete Freund's adjuvant and to emphasize the apparent selective destruction of cells with abnormal growth characteristics (*e.g.*, tumor cells and an established cell line) by these activated macrophages.

Materials and Methods. Female C_3H/HeJ mice weighing 20–25 g were purchased from Jackson Memorial Laboratory, Bar Harbor, MA. Strain of *T. gondii* and methods of handling have been described (10). To establish chronic infection mice were infected with 5×10^3 trophozoites of the C_{56} strain of *T. gondii*; sulfadiazine started 4 days later was continued for 20 days. One month prior to collection of peritoneal macrophages for use in the cytotoxicity test, chronically infected mice were challenged ip with 5×10^4 trophozoites of the virulent RH strain of *T. gondii*. CFA and incomplete Freund's adjuvant (ICFA) (Difco Laboratories, Detroit, MI) were prepared and inoculated as described in the companion paper (9). A third group of mice were injected with Hanks' balanced salt solution (HBSS). Peritoneal cells for use in the *in vitro* cytotoxicity test were

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[3] We define abnormal growth as the ability of cells to develop into a malignant tumor when injected into an appropriate host or, if transformed *in vitro*, to have an unlimited proliferation in tissue culture, *i.e.*, to develop into an established cell line as defined by Hayflick and Moorhead (4). An established cell line would fit our definition of cells with abnormal growth characteristics if they also have lost density dependent inhibition of growth (5) as well as contact inhibition of movement (6). It has been shown that most established cell lines develop malignant properties over the course of long-term serial culture (7). Cells with normal growth characteristics would, by the criteria of Hayflick and Moorhead (4), be termed a cell strain.

obtained as previously described (10). 2×10^6 cells in 0.1 ml of HBSS from mice pretreated with CFA, ICFA or HBSS were pipetted onto sterile 24 × 16-mm coverslips which had been placed in 35 × 10 mm-tissue culture dishes (Falcon Plastics). The dishes were incubated for 2 hr at 37° in a 5% CO_2 atmosphere to allow for adherence of cells. The coverslips were then washed 5 times with a total of 20 ml of sterile normal saline to remove nonadherent cells. At this time, target cells were added. Target cells and their method of preparation have been described (3). A suspension of target cells 5×10^4 in 0.1 ml of medium (M 199 with 20% fetal calf serum and 100 units penicillin G plus 100 μg streptomycin/ml) was added to coverslips containing the washed monolayers of adherent cells from which medium had been removed. Additional medium was added to dishes after allowing the target cells to settle onto the adherent peritoneal cells for 20 min, and the tissue culture dishes containing mixed macrophages and target cells incubated for 60 hr at 37°. The coverslips were then fixed in methanol and stained with Giemsa stain. Purity of macrophages in control adherent cell monolayers was determined by phagocytosis of heat-killed *Candida albicans*.

Results. The *in vitro* interaction of target cells with peritoneal macrophages from C_3H/HeJ (H-2k) mice pretreated 7 weeks earlier with CFA, ICFA or HBSS was evaluated. EMT-6 mammary adenocarcinoma (H-2d) which developed spontaneously in a Balb/C mouse (11), Balb/C mouse embryo cells (H-2d) and C_3H/HeJ mouse embryo cells (H-2k) were employed as target cells. Activated macrophages from C_3H/HeJ mice pretreated with CFA were cytotoxic for EMT-6 tumor cells but not for Balb/C mouse embryo or C_3H/HeJ mouse embryo cell strains. Macrophages from ICFA and HBSS pretreated mice were not cytotoxic for any of the target cells employed. Figure 1 is representative of the gross plaquing which occurred on coverslips where CFA activated macrophages and EMT-6 mammary adenocarcinoma cells were mixed. It should be noted that CFA activated macrophages did not cause gross plaquing to either syngeneic or allogeneic mouse embryo cell strains and that there was no microscopic evidence of destruction. Macrophages from ICFA and HBSS controls were not cytotoxic to any of the target cells including EMT-6 mammary adenocarcinoma cells. Results were compared to those obtained with macrophages from mice with chronic *T. gondii* (3) infection and are summarized in Table I. Cytopathic effect was noted only in cultures where macrophages activated by either chronic *T. gondii* infection of CFA were in contact with target cells with abnormal growth characteristics, *i.e.*, KHT sarcoma cells, EMT-6 mammary adenocarcinoma cells, or L cells. Normal mouse embryo cells were not destroyed regardless of their histocompatibility type. Monolayers of adherent peritoneal cells used in these *in vitro* studies were determined to be greater than 99% macrophages by phagocytosis of heat-killed *Candida albicans*.

Discussion and Summary. In 1964 it was reported that specifically immune macrophages could destroy allogeneic target cells *in vitro* by a nonphagocytic mechanism (12). More recently, similar but nonimmunologic destruction of tumor target cells by activated macrophages has been described (1, 3). The present study demonstrates that activated macrophages from mice pretreated with CFA 7 weeks previously are cytotoxic to cells with abnormal growth characteristics *in vitro*. The results parallel those obtained with another system, activated macrophages from mice chronically infected with *T. gondii* (3).

The following points characterize the activated macrophage mediated cytotoxic reaction as we have observed it in this and other studies:

(1) The effector cell appears to be the activated macrophage. L cells were not destroyed by purified preparations of lymphocytes from mice whose activated peritoneal macrophages were cytotoxic to L cells or by nonactivated macrophages (3).

(2) Intimate contact between activated macrophages and target cells is apparently necessary for the cytotoxic reaction to occur. No cytopathic effect was noted when cell free

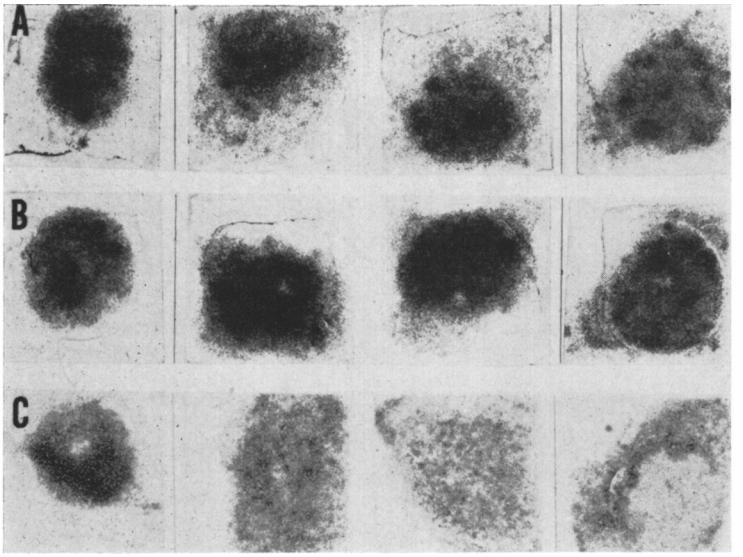

FIG. 1. A = C_3H mouse embryo cells; from left to right; target control; macrophage monolayer from HBSS controls plus target cells; macrophage monolayer from ICFA controls plus target cells; macrophage monolayer from CFA inoculated mice plus target cells; B = Balb/C mouse embryo target cells; same sequence; C = EMT-6 mammary adenocarcinoma cells; same sequence. In all cases 2×10^6 peritoneal cells were added to sterile coverslips and all nonadherent cells removed by washing 5 times with sterile normal saline; 5×10^4 target cells were added to each macrophage monolayer; the cultures incubated for 72 hr in 5% CO_2 and 95% air; and finally fixed in methanol and stained with Giemsa stain.

medium, taken from cultures in which activated macrophages had mediated destruction of L cells, was added to L cell monolayers growing alone (3).

(3) Activation of macrophages results in increased *in vitro* nonspecific microbiocidal capacity for intracellular pathogens (10, 13) and in increased *in vitro* nonspecific cytotoxic potential for cells with abnormal growth properties (3). Furthermore, our studies show that agents which activate macrophages can stimulate nonspecific resistance to both an intracellular organism (*e.g., Listeria monocytogenes*) as well as neoplasia (2, 3, 9).

(4) Activation of macrophages can be effected by diverse agents, living as well as nonliving. Activation of macrophages with increased cytotoxic capabilities was stimulated

TABLE I. Effect of Activated Macrophages on Target Cells *in Vitro*.

Effector cells	Target cells					
Peritoneal macrophages from C_3H/HeJ female (H-2k) mice	Sarcoma KHT (H-2k)	L cells (H-2k)	C_3H/HeJ female mouse embryo cells (H-2k)	EMT-6 adenocarcinoma (H-2d)	Balb/c mouse embryo cells (H-2d)	C_{57}BL/6J mouse embryo cells (H-2b)
Chronic *T. gondii* infection	+[a]	+	0	+	0	0
Pretreated with CFA	N.D.[b]	+	0	+	0	N.D.
Pretreated with ICFA	N.D.	0	0	0	0	N.D.
Pretreated with HBSS	0[c]	0	0	0	0	0

[a] + = Cell destruction.
[b] N.D. = Not done.
[c] 0 = No destruction.

in mice by chronic infection with intracellular protozoa (3), infection with an intracellular bacteria (3), and in this study with CFA.

Due to the similarity between the nonspecific resistance to neoplasia stimulated by chronic intracellular infection (2) and that stimulated by agents with properties which include immunologic adjuvant activities (9), we believe that the many agents which have been used to nonspecifically increase host resistance to tumors may operate, in part at least, through the common mechanism of stimulating activation of host macrophages with increased cytotoxic activity (3). Independently, Alexander and Evans noted macrophage mediated nonspecific destruction of tumor cells following macrophage activation with poly I:poly C and endotoxin (14). These results and our own suggest that macrophage activation can be effected nonimmunologically as well as develop as a by-product of a specific immunologic event.

(5) The efferent limb of the activated macrophage mediated cytotoxic reaction appears to be specific but not in an immunologic sense. The discriminatory ability of activated macrophages does not appear to be based on recognition of foreign target cell antigens but apparently on recognition of a cellular property associated with abnormal growth (3). The activated macrophage can apparently distinguish normal cells from cells with abnormal growth properties because only the latter are destroyed (3). This suggests that activated macrophage mediated cytotoxic reactions may represent a nonimmunologic mechanism of importance in the homeostatic control of abnormal cell growth in normal hosts.

1. Hibbs, J. B., Jr., Lambert, L. H., Jr., and Remington, J. S., J. Clin. Invest. **50**, 45a (1971).

2. Hibbs, J. B., Jr., Lambert, L. H., Jr., and Remington, J. S., J. Infect. Dis. **124** (1971).

3. Hibbs, J. B., Jr., Lambert, L. H., Jr., and Remington, J. S., Nature New Biol. **235**, 48 (1972).

4. Hayflick, L., and Moorhead, P. S., Exp. Cell Res. **25**, 585 (1961).

5. Stoker, M. G. P., and Rubin, H., Nature (London) **215**, 171 (1967).

6. Abercrombie, M., and Heaysman, J. E. M., Exp. Cell Res. **6**, 293 (1954).

7. Rothfels, H. H., Kupelwieser, E. B., and Parker, R. C., *in* "Canadian Cancer Conference" (R. W. Beggs *et al.*, eds.), Vol. 5, p. 191. Academic Press, New York (1963).

8. Ruskin, J., and Remington, J. S., J. Reticuloendothel Soc. **9**, 465 (1971).

9. Hibbs, J. B., Jr., Lambert, L. H., Jr., and Remington, J. S., Proc. Soc. Exp. Biol. Med. **139**, 1053 (1972).

10. Ruskin, J., and Remington, J. S., Science **160**, 72 (1968).

11. Rockwell, S. C., Ph.D. thesis, Stanford University (1971).

12. Granger, G. S., and Weiser, R. S., Science **145**, 1427 (1964).

13. Mackaness, G. B., J. Exp. Med. **120**, 105 (1964).

14. Alexander, P., and Evans, R., Nature New Biol. **232**, 76 (1971).

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