posterior lateral teeth (Figs. 1D and 2); it is not known whether cardinal teeth were present. The posterior hinge area is concave and nearly vertical; there is no sign of nymphs or ligamental grooves, which suggests that the ligament was a simple structure consisting of inner and outer layers connecting the two valves. The outer layer would connect the valve edges along the dorsal midline; below this and attached to the concave hinge area would be the inner part of the ligament.

The shape of *Fordilla troyensis* suggests that it is allied to the Ordovician actinodontoid heteroconchs or the Isofilibranchia; the known musculature is consistent with assignment to either group. Possibly the actinodontoids and isofilibranchs may be related to each other through *Fordilla*. Because of the lack of anterior and posterior lateral teeth it seems unlikely that *Fordilla* can be treated as a direct ancestor of the palaeotaxodonts or the pteriomorphs of the Ordovician. The former group has taxodont dentition which extends along the length of the hinge, and the latter group usually has prominent posterior lateral teeth. The remaining subclass of Ordovician pelecypods, the anomalodesmatans, is composed of elongate burrowing forms of quite different shape from *Fordilla*. Tentatively, it is suggested that *Fordilla* gave rise to the Actinodontoida and the Isofilibranchia and that the Palaeotaxodonta, Pteriomorphia, and Anomalodesmata arose from the Isofilibranchia or the Actinodontoida (*13*).

JOHN POJETA, JR.
*U.S. Geological Survey,*
*Washington, D.C. 20242*
BRUCE RUNNEGAR
*University of New England,*
*Armidale, New South Wales, Australia*
JIRI KRIZ
*Central Geological Survey,*
*Prague, Czechoslovakia*

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17. J.P. publishes with the permission of the director, U.S. Geological Survey; B.R.'s contribution was prepared while on study leave from the University of New England and was supported by a grant from the Smithsonian Institution; J.K.'s contribution was prepared during the tenure of a Smithsonian Institution postdoctoral fellowship. Figured specimens are on deposit at the U.S. National Museum, Washington, D.C. (Fig. 1, A, B, and D), or the New York State Museum, Albany (Fig. 1C).

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## Macrophage Nonimmunologic Recognition: Target Cell Factors Related to Contact Inhibition

Abstract. *Activated mouse macrophages were not cytotoxic to contact-inhibited nontumorigenic 3T3 fibroblasts, but caused marked destruction to non-contact-inhibited, tumorigenic 3T12 and simian virus 40–transformed fibroblasts. Nonimmunologic recognition and destruction of target cells by activated macrophages is independent of altered morphology, abnormal karyotype, and ability for continuous multiplication in vitro—all characteristics of 3T3 fibroblasts. A modification of the target cell surface that results in a high in vitro saturation density, agglutinability by plant lectins, and tumorigenicity appears to evoke a cytotoxic response by activated macrophages.*

Activated macrophages recognize and nonspecifically destroy in vitro target cells with abnormal growth properties by a nonphagocytic mechanism (*1*, *2*). The present results suggest the abnormal growth characteristic important in nonimmunologic macrophage recognition as a target cell surface membrane modification associated with loss of contact inhibition. The important role of the surface membrane in control of cell behavior is indicated by the in vitro phenomenon of contact inhibition of mitosis (*3*) and movement (*4*). Loss of contact inhibition at confluency in tissue culture reflects a fundamental change in sensitivity to short-range cellular growth control signals and can be correlated with tumorigenicity (*5*). The selective recognition and response by activated macrophages to target cells that have lost contact inhibition could be the in vitro correlate of a host defense mechanism directed against cell surface changes associated with neoplastic growth.

In order to show the importance of surface factors in the nonimmunologic cytotoxicity reaction between activated macrophages and target cells, the following cell lines were evaluated: BALB/c 3T3 fibroblasts (a contact-inhibited nontumorigenic cell line), BALB/c 3T12 fibroblasts (a non-contact-inhibited, tumorigenic cell line from the same original pool of mouse cells as the BALB/c 3T3 line), and simian virus 40–transformed BALB/c 3T3 fibroblasts (SV-3T3 cells—a non-contact-inhibited tumorigenic cell line) (*6*). The results show that 3T3 fibroblasts were not destroyed while 3T12 and SV-3T3 fibroblasts were destroyed by syngeneic and allogeneic activated macrophages. This suggests that surface alterations related to loss of contact inhibition, a property of the 3T12 and SV-3T3 lines, is an important factor in target cell recognition and destruction. Abnormal characteristics of the 3T3 fibroblast line that appear not to play a role in nonimmunologic recognition and destruction by activated macrophages include (i) loss of normal cell morphology, (ii) hypotetraploid karyotype, (iii) maximum growth in vitro to confluency from a very low inoculum, and (iv) continuous multiplication in vitro (*6*). In addition, activated macrophages were not cytotoxic to secondary cultures of mouse kidney cells (MKC) but were cytotoxic to EMT-6 mouse mammary adenocarcinoma cells, suggesting a similar mechanism of recognition of contact-inhibited epithelial cells as for contact-inhibited fibroblasts.

Immunologic activation in vivo of macrophages was produced by infecting 6-week-old C3H/He female mice with the $C_{56}$ strain of *Toxoplasma gondii* as previously described (*2*) or by infecting 6-week-old female C3H/He or BALB/c mice with the Paris strain of Bacillus Calmette-Guérin (BCG). BCG [0.2 mg (wet weight) in

0.2 ml of 0.15*M* NaCl] was injected intraperitoneally. One month after the primary infection was established, the mice received a booster injection of the same dose of BCG intraperitoneally. Peritoneal cells were harvested for use in the cytotoxicity test 2 months after chronic infection with toxoplasma was established or 3 to 18 days after the booster inoculation of BCG was given.

In order to determine whether endotoxin activated macrophages, which are cytotoxic to tumor cells (*7*), are capable of nonimmunologic discrimination between contact-inhibited and non-contact-inhibited target cells, we exposed macrophage monolayers from normal BALB/c female mice in vitro to endotoxin (*Escherichia coli* lipopolysaccharide, Difco) in concentrations of 1.0 to 40 $\mu$g per milliliter of medium. Normal macrophages were activated by prior treatment in vitro with endotoxin for 12 to 24 hours before addition of the target cells and by continual exposure to endotoxin after the target cells had been added to the macrophage monolayers. Endotoxin in the concentrations used caused no inhibition of target cells growing alone.

Macrophages activated either in vivo or in vitro caused little or no cytotoxicity to fibroblast or epithelial contact-inhibited target cells including the BALB/c 3T3 line (Table 1). However, the same syngeneic or allogeneic activated macrophages were cytotoxic to non-contact-inhibited tumorigenic target cells (*8*). Normal macrophages were not cytotoxic to either contact-sensitive or non-contact-sensitive target cells. Figure 1 shows the effect of normal BALB/c macrophages and BALB/c macrophages activated by BCG infection (peritoneal cells harvested 2 weeks after the mice were given a booster inoculation of BCG) on BALB/c 3T3 and BALB/c 3T12 fibroblasts.

Saturation density measurements were made on the target cells used in the cytotoxicity test in order to establish their growth kinetics in vitro. The cells were inoculated in triplicate at a density of $1 \times 10^5$ in 25 cm$^2$ plastic tissue culture flasks (Falcon) and incubated for 3 days; the medium was changed on day 3, and the cells were grown for 3 days more and then detached with trypsin and counted (Table 1). Target cell destruction by activated macrophages correlated with high saturation densities. Growth kinetics of the MKC could not be studied because, at the low inoculum used, there was fibroblast overgrowth after 6 days of culture. When MKC were used in the cytotoxicity test, a larger number of cells was inoculated per unit of tissue culture surface and, although there was some fibroblast growth, numerous islands of epithelial cells grew among both normal and activated macrophages.

To demonstrate that increased in vitro nonspecific cytotoxic activity for non-contact-inhibited cells and increased nonspecific microbicidal activity for intracellular pathogens are parallel phenomena, monolayers of normal and activated macrophages from the same groups of mice used as a source of peritoneal cells for the cytotoxicity test were challenged with $5 \times 10^5$ trophozoites of the RH strain of toxoplasma obtained from the peritoneal fluid of a mouse infected 3 days earlier (*9*). Activated macrophages from mice chronically infected with the $C_{56}$ strain of toxoplasma and from BCG infected mice and normal macrophages activated in vitro with endotoxin were capable of resisting destruction when compared to macrophages from control mice. In addition, fewer RH toxoplasma trophozoites were released from activated macrophages than from normal macrophages at 36 hours after infection. For example, the average number of trophozoites released from four monolayers of BCG activated macrophages was $6.7 \times 10^4 \pm 2.5 \times 10^4$ and from the average of four monolayers of normal macrophages $1.8 \times 10^6 \pm 1.5 \times 10^5$. These results and those of the cytotoxicity test are evidence that diverse methods of macrophage activation (chronic toxoplasma infection, chronic BCG infection, and endotoxin) produce a similar enhancement of macrophage microbicidal and cytotoxic activity. Macrophage activation with cytotoxic activity for non-contact-inhibited target cells may explain, in part at least, the mechanism of action of BCG as a nonspecific immunotherapeutic agent in the treatment of human cancer.

Surface changes that occur in cells with neoplastic potential are antigenic and can induce the development of a specific immune response (*10*); and macrophages are capable of participation in this immune response (*11*). However, the surface modification of

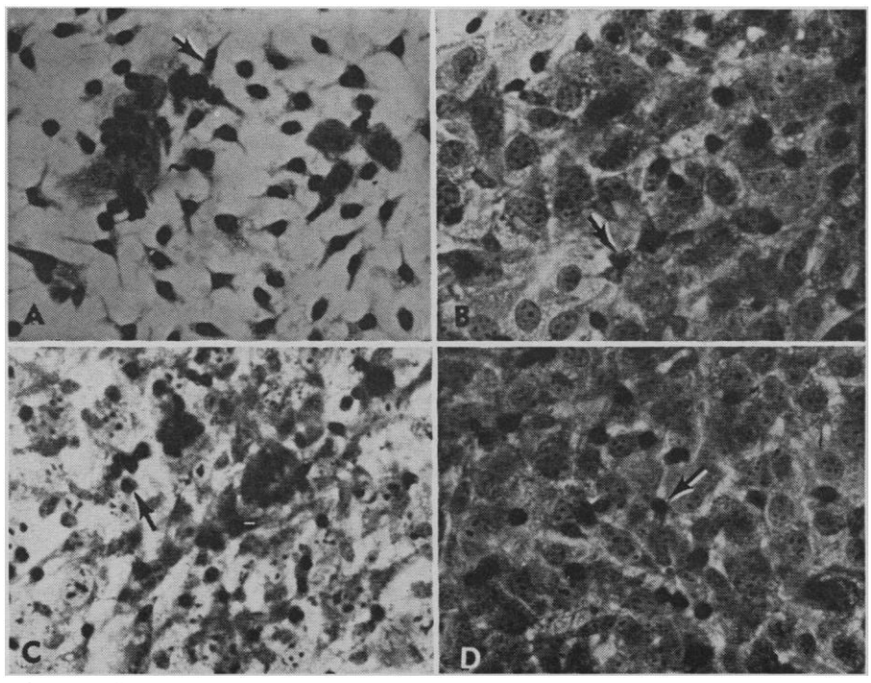


Fig. 1. Effect of normal and BCG activated BALB/c macrophages on contact-inhibited BALB/c 3T3 fibroblasts and non-contact-inhibited BALB/c 3T12 fibroblasts. Arrows point to macrophages. Protocol for the cytotoxicity test is described in the legend to Table 1. (A) Marked destruction of 3T12 fibroblasts by activated macrophages. Smaller activated macrophages can be observed in contact with larger 3T12 fibroblasts. Fibroblast in contact with the activated macrophage marked with the arrow is pycnotic and degenerating. (B) Activated macrophages cause no cytopathic effect to 3T3 fibroblasts. (C) Normal macrophages are overgrown by 3T12 fibroblasts. (D) Normal macrophages have no cytotoxic effect on 3T3 fibroblasts (× 750).

Table 1. Effect of activated macrophages on contact-inhibited and non-contact-inhibited target cells and measurement of growth kinetics of target cells. Target cells were cultured in Dulbecco's modification of Eagle's medium with 10 percent fetal calf serum (Gibco), streptomycin (100 $\mu$g/ml), and penicillin (100 unit/ml). Primary cultures of mouse embryo fibroblasts (MEF) were prepared from 17- to 19-day embryos, and primary cultures of MKC were prepared from weanling mice. MEF and MKC were prepared as described (*17*). The BALB/c mouse mammary adenocarcinoma (EMT-6) line has been described (*18*). Peritoneal cells ($7.0 \times 10^5$) (*16*) were added to chambers of Lab Tek (Miles Laboratories) eight-chambered tissue culture slides. The slides were incubated for 2 hours at 37°C in air containing 5 percent $CO_2$ to allow for adherence of cells. Each chamber containing peritoneal cells was washed six times with Hanks balanced salt solution to remove nonadherent cells. A suspension of target cells (*2*), $3 \times 10^4$ cells in 0.2 ml of medium, was added to the Lab Tek chambers containing the washed monolayers of adherent peritoneal cells (macrophages). The target cells were allowed to settle onto the adherent macrophages for 2 to 3 hours, and the tissue culture slides containing mixed macrophages and target cells were incubated in Dulbecco's modification of Eagle's medium with 20 percent fetal calf serum (Gibco), streptomycin (100 $\mu$g/ml), and penicillin (100 unit/ml) for 60 hours at 37°C in atmosphere containing 5 percent $CO_2$. The tissue culture slides were then fixed in methanol and stained with Giemsa. Symbols used: 0, little or no target cell destruction; +, marked target cell destruction.

| Target cells | Source of effector cells [C3H/He (H-$2^k$) and BALB/c (H-$2^d$) peritoneal macrophages] | | | | | Target cell growth kinetics† |
|---|---|---|---|---|---|---|
| | Control BALB/c | Control C3H/He | BALB/c BCG | C3H/He *T. gondii* | BALB/c activated with endotoxin* | |
| | | | *Contact-inhibited cells* | | | |
| BALB/c 3T3 | 0 | 0 | 0 | 0 | 0 | 11 |
| BALB/c MEF | 0 | 0 | 0 | 0 | 0 | 6.5 |
| C3H/He MEF | 0 | 0 | 0 | 0 | 0 | 8.0 |
| BALB/c MKC | 0 | 0 | 0 | 0 | 0 | |
| C3H/He MKC | 0 | 0 | 0 | 0 | 0 | |
| | | | *Non-contact-inhibited tumorigenic cells* | | | |
| BALB/c 3T12 | 0 | 0 | + | + | + | 61 |
| BALB/c SV-3T3 | 0 | 0 | + | + | + | 157 |
| BALB/c EMT-6 | 0 | 0 | + | + | + | 96 |

* *Escherichia coli* lipopolysaccharide B (Difco 0.55 : B5). † Ratio of number of cells in culture after 6 days to the number of cells inoculated.

non-contact-inhibited cells may be so extensive that it can evoke nonimmunologic recognition and response by activated macrophages (even though the animal had not been sensitized to the target cell).

There is evidence that extensive modification of the cell surface does occur after loss of contact inhibition (*5, 12, 13*). Others working with the 3T3, 3T12, and SV-3T3 series of mouse fibroblasts have shown that tumorigenicity (*5*) and cell agglutination after plant lectin binding (*12*) correlate with loss of contact inhibition. Likewise, I have shown that nonimmunologic recognition and destruction by activated macrophages are also associated with loss of contact inhibition by these target cells. All of these findings may be related to extensive biochemical or topographical differences (or both) in the surface membrane of contact-inhibited (3T3) and non-contact-inhibited (SV-3T3) fibroblasts (*13*). In addition to being a possible mechanism for the control of abnormal cell growth, this cytotoxicity system could serve as a prototype model for the involvement of macrophages in certain types of programmed cell destruction (*14*).

The results of these experiments might have relevance to the effective in vivo functioning of activated macrophages during certain infections. Macrophage activation is an important homeostatic event; local or systemic populations (or both) of activated macrophages are probably essential to the destruction and control of many, if not all, intracellular pathogens (*15, 16*). Since, in situ, activated macrophages are often a component of normal tissues, it is essential that they not be nonspecifically cytotoxic to cells with unmodified surfaces. The present study demonstrates that activated macrophages, with increased nonspecific microbicidal activity for an intracellular pathogen (toxoplasma) and nonspecific cytotoxic activity for non-contact-inhibited cells, do not destroy cells with normal surfaces.

JOHN B. HIBBS, JR.
*Veterans Administration Hospital* and
*Department of Medicine,*
*Division of Infectious Diseases,*
*University of Utah Medical Center,*
*Salt Lake City 84112*

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