Reports

Homograft Target Cells: Specific Destruction in vitro by Contact Interaction with Immune Macrophages

Abstract. *Specific adherence and mutual immunologic destruction occurred after contact in vitro of immune peritoneal macrophages from C57Bl/6K mice with homograft target cells from A/Jax mice. Cell destruction apparently resulted from a nonphagocytic mechanism involving cell contact rather than humoral antibody. Cell destruction was determined by a plaque technique with either immune cells or target cells as monolayers.*

The specific capacity of immune lymphocytes to destroy homologous target cells by close association in vitro has been demonstrated by several investigators (*1*, *2*). In some instances the immune lymphocytes as well as the target cells have been killed in the interaction (*2*).

With the exception of Weaver's brief report on the immune macrophage (*3*), the lymphocyte is the only cell type known that can, in vitro, bring about the immunologic destruction of target cells by a nonphagocytic mechanism. However, several investigators have reported that immune macrophages have the specific capacity to destroy homografted cells in vivo (*4*, *5*), presumably through some mechanism of contact interaction or close association. There is evidence that, in the interaction of immune macrophages with target cells in vivo, the macrophages as well as the target cells may be destroyed (*4*, *5*), a phenomenon called "allergic death" by Gorer and Boyse (*6*).

Our studies on the capacity of immune macrophages to bring about "contact destruction" of target cells in tissue culture monolayers follow previous observations that an acute form of allogeneic disease of mice may result from the destructive activities of immune macrophages after contact with target cells of the host (*5*).

The experiments were designed to give evidence of the interaction of immune macrophages and target cells by inducing plaques within a monolayer. In some instances target cells, either normal macrophages or fibroblasts, served as the monolayer and in other instances immune macrophages served as the monolayer. Plaques were induced by depositing an overlay of "reacting cells" at loci on the monolayer.

Immune peritoneal cells were obtained from the peritonea of C57B1/6K mice 10 days after an intraperitoneal homograft of 20 million Sarcoma I (SaI) ascites tumor cells derived from the host of origin, the A/Jax mouse. The macrophage-rich ascitic fluids were aspirated, pooled, and centrifuged in the cold at 50*g* for 4 minutes. The suspensions were kept at 4°C during preparation in order to minimize spontaneous agglutination. The sedimented cells were washed four times in cold Hanks balanced salt solution (HBSS) containing 1 percent normal mouse serum and resuspended in a standard medium consisting of a base medium containing HBSS 90 percent, yeast extract 0.1 percent, proteose peptone No. 3 0.1 percent, penicillin 100 units per milliliter, streptomycin 100 μg/ml, and 10 percent calf serum. Examination of the cell suspension by phase microscopy and the May-Grunwald-Giemsa staining method showed that it was essentially devoid of tumor cells and that it was composed of 90 to 95 percent macrophages, 3 to 5 percent lymphocytes, and a few miscellaneous cells. Cell counts were made and the volume of each suspension was adjusted to give a concentration of approximately 1 million cells per milliliter.

Normal peritoneal cells from A/Jax mice were collected 12 hours after the last of three intraperitoneal doses, of 4 ml each, of 1 percent oyster glycogen in physiological saline given on alternate days. The cells were washed and suspended as we have already described for immune peritoneal cells. The preparation contained 85 percent macrophages and 12 percent lymphocytes. They showed less tendency to clump spontaneously than did immune cells.

Monolayers were prepared by adding cell suspensions to small tissue culture bottles to attain a fluid depth of 6 mm over an area of 18 cm^2. The monolayers were allowed to develop for 12 hours at 37°C under an atmosphere of 5 percent CO_2 before use. The tissue culture medium was replaced at 12 hours, 24 hours, and daily thereafter.

Fibroblasts used as target cells were obtained by treating whole minced mouse fetuses with 0.25 percent trypsin for several hours at 37°C. The cells were grown in the standard culture medium for 48 hours at 37°C under 5 percent CO_2. The medium was discarded, and the fibroblasts were removed from the glass by treatment with 0.05 percent trypsin for 15 minutes. Standard culture medium was added and monolayers were allowed to develop in small tissue culture bottles with or without coverslips.

The suspensions of "overlay cells" to be superimposed on monolayers were adjusted to give 10 million cells per milliliter. To establish foci of overlay cells the medium on the monolayers was first discarded and replaced with fresh medium to a depth of 2 mm. The suspension of cells was then drawn into a capillary pipette, and a small drop containing approximately 250,000 cells was deposited at each desired site over the monolayer. The cells settled on the monolayer, and remained within an area approximately 1 cm in diameter. The monolayers were examined after 6, 12, 24, 48, and 60 hours of incubation.

Since strong adherence was noted to occur between immune cells and target cells, monolayers of A/Jax fibroblasts, C57B1/6K fibroblasts, and HeLa cells were prepared and then treated with overlay preparations of immune and normal C57B1/6K peritoneal cells. Attempts to remove the overlay cells by washing with physiological saline solution showed that within 4 minutes the immune cells displayed strong adherence limited to their specific target cells, the A/Jax fibroblasts. This was evident microscopically and was grossly visible by the virus-plaque staining method (*7*). "Nonspecific" adherence in control preparations was observed but was limited to a few cells, and it

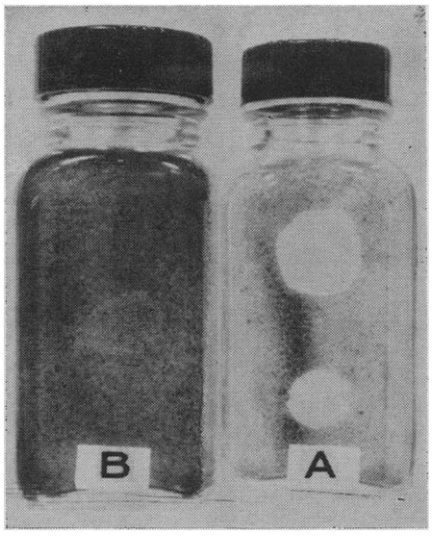

Fig. 1. *A*, Two 48-hour plaques of complete clearing produced on a monolayer of A/Jax fibroblasts with peritoneal cells from C57B1/6K mice immunized with Sarcoma I. *B*, A 24-hour plaque of partial clearing produced on a monolayer composed of equal numbers of HeLa cells and L cells with peritoneal cells from C57B1/6K mice immunized with Sarcoma I.

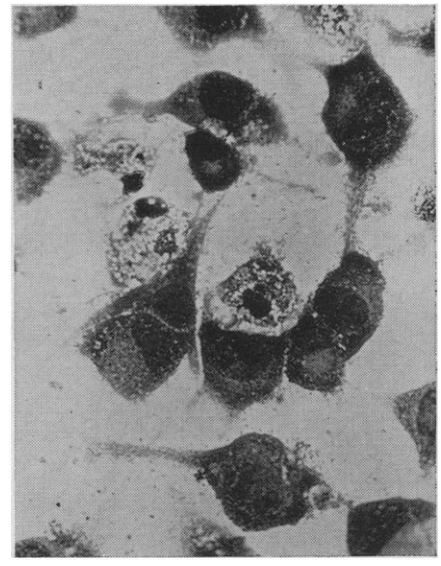

Fig. 2. Monolayer of L cells and superimposed peritoneal cells from C57B1/6K mice immunized with Sarcoma I, at 3.5 hours. The macrophages, which are in intimate contact with L cells, are easily identified by numerous clear intracytoplasmic lipid droplets

did not become evident before 30 minutes.

In the first experiment immune peritoneal cells were deposited at loci on monolayers of normal A/Jax peritoneal macrophages employed as specific target cells. The first evidence of immune cell–target cell interaction was cell adherence which became apparent within 4 minutes. Most of the cells at the site of the immune cell overlay exhibited injury at 12 to 24 hours as indicated by vital staining with 0.1 percent eosin Y. Detachment of macrophages from the glass was evident at 24 hours, and by 60 hours a plaque could be visualized by the virus-plaque staining method. The few viable macrophages which remained attached to the glass in the plaque area could not be identified as being either immune cells or target cells. However, it was obvious that most of the immune cells, as well as the target cells, were destroyed as the result of their interaction. Control monolayers of normal C57B1/6K macrophages treated with immune peritoneal cells showed a limited amount of "nonspecific" cell adherence and no evidence of cell injury or plaque formation.

In other experiments in which the reverse procedure was performed, that is, when normal A/Jax peritoneal cells were deposited on lymphocyte-free monolayers of immune macrophages, plaques resulted as before. No plaques developed in control preparations when normal peritoneal cells from C57B1/6K or DBA/2 mice, which are similar at the H_2 locus, were deposited on monolayers of immune macrophages.

In the next experiments coverslip preparations were used to facilitate better study of cell interaction and cytology by the use of stains and higher magnifications.

Immune peritoneal cells were washed six times in HBSS containing 1 percent mouse serum and placed at loci on coverslip monolayers of fetal A/Jax fibroblasts as specific target cells. The preparations were stained by the May-Grunwald-Giemsa method after 6, 12, 24, 48, and 60 hours of incubation. Marked aggregation of the immune cells around the target cells became apparent at 6 to 12 hours after inoculation. At the 12th hour both immune cells and target cells were rounded, and their cytoplasmic membranes were indistinct, particularly at points where immune cells and target cells were in contact. At 24 hours the nuclei showed pyknosis and karyorrhexis. Plaques were fully developed after 48 to 60 hours as shown in Fig. 1*A*. A few sparsely scattered immune macrophages remained adhering to the glass in the plaque area. In control preparations, in which immune peritoneal cells were deposited on monolayers of C57B1/6K fibroblasts or on Balb/C fibroblasts, no cell injury was observed even though the immune cells adhered nonspecifically to the monolayer. Other control preparations in which normal peritoneal cells from C57B1/6K mice were deposited on monolayers of A/Jax fibroblasts did not show cell injury.

The reverse procedure of depositing A/Jax fibroblasts on lymphocyte-free monolayers of immune macrophages yielded plaques.

To test the action of humoral antibody alone on target cells, coverslip monolayers of A/Jax fibroblasts were incubated for 1 hour at 37°C in the base tissue culture medium supplemented with 20 percent immune antiserum to SaI from C57B1/6K mice. The immune serum was prepared by 8 weekly intraperitoneal injections of 20 million SaI cells, and its titer against A/Jax red cells was 2^{14} as determined by the hemagglutination method of Stimpfling (*8*). In the presence of rabbit complement it showed a cytotoxicity of 70 percent for SaI cells at a dilution of 2^{10}. The culture fluid containing immune serum was replaced with standard culture medium containing 10 percent heat-inactivated calf serum and incubation was continued at 37°C. After intervals of 2 and 12 hours the cells were fully viable as determined by vital staining with eosin Y. Likewise, May-Grunwald-Giemsa stains revealed no evidence of cell injury after intervals of 24, 48, and 60 hours.

The possibility that target cells coated with antibody would be injured by normal peritoneal cells was explored. Monolayers of A/Jax fibroblasts were incubated in the basic culture medium supplemented with 20 percent immune serum for 1 hour at 37°C. This culture fluid was replaced with standard culture medium, and normal C57B1/6K peritoneal cells were deposited at foci on the monolayer. No evidence of cell injury was detected in these cultures even as late as 72 hours.

In order to determine whether normal peritoneal cells would behave as "immune cells" in the presence of excess humoral antibody, normal C57B1/6K peritoneal cells were deposited at foci on monolayers of A/Jax fibroblasts in base culture medium supplemented with 20 percent immune serum. Examination of the culture after intervals of 24, 48, and 60 hours revealed no evidence of target cell injury.

The specificity of the action of im-

mune macrophages on target cells was further demonstrated by their failure to form plaques on monolayers of HeLa cells and their capacity to form plaques on monolayers of L cells from C3H mice. Both C3H mice and A/Jax mice possess antigen or antigens determined by the *K* allele of the H_2 locus, an allele which is lacking in C57B1/6K mice.

The possibility that control "nonspecific target cells" might be injured if placed in close association with interacting immune cells and specific target cells was explored by depositing immune cells at foci on a mixed monolayer consisting of HeLa and L cells in ratios of 5 : 1, 4 : 1, 3 : 1, 2 : 1, 1 : 1, 1 : 2, 1 : 3, 1 : 4, and 1 : 5. The plaque of partial clearing shown in Fig. 1*B* was produced on a monolayer comprised of equal numbers of L cells and HeLa cells. The plaque which formed in 24 to 48 hours appeared to have resulted from the complete destruction of the L cells without injury to the HeLa cells. Whereas essentially all of the L cells in the foci were killed regardless of the ratio of HeLa cells to L cells, the HeLa cells did not show evidence of injury. The transparency of the plaques correlated directly with the proportion of L cells present in the monolayer. Grossly visible plaques were evident at ratios of HeLa cells to L cells between 2 : 1 to 1 : 5.

The influence of increasing the ratio of immune cells to target cells by holding the immune cells constant and varying the density of the monolayers of A/Jax fibroblasts and monolayers of L cells was investigated. The results showed that the target cells in monolayers of low density were rapidly destroyed during the period extending from 1 to 6 hours. This rapid destruction of target cells in monolayers of low density was presumed to take place because of the large numbers of immune cells which made contact with the target cells in such preparations. More than 95 percent of the adhering cells were macrophages. Whereas only an occasional lymphocyte was adherent to the target L cells, most lymphocytes were free. Contact between macrophages and target cells is shown in Fig. 2.

The following additional observations have been made. Plaques are formed on monolayers of A/Jax fibroblasts by: (i) peritoneal cells derived from C57B1/6K animals immunized by the intraperitoneal injection of A/Jax spleen cells, (ii) peritoneal cells derived from C57B1/6K animals immunized subcutaneously with SaI, (iii) immune macrophages purified by the iron magnet method of Rous and Beard containing 96 percent macrophages and 2 percent lymphocytes (*9*). Lymphocyte-rich preparations obtained from ascitic exudates of SaI-immunized animals by repeated slow-speed centrifugation in the standard tissue culture medium destroyed a limited number of target cells in monolayers of A/Jax fibroblasts but did not produce visible plaques. The final lymphocyte-rich supernatant fluid contained 73 percent lymphocytes and 25 percent small macrophages.

In none of the cytologic studies was there any evidence that target cell injury resulted from phagocytosis by immune cells.

The results establish that peritoneal macrophages from C57B1/6K mice immunized with Sarcoma I ascites tumor produce specific immunologic injury of the target cells, macrophages and fibroblasts, by a nonphagocytic mechanism which apparently does not depend on humoral antibody, but instead demands contact between immune cell and target cell. The phenomenon probably involves antigen(s) determined by the K allele of the H_2 locus and is distinct from the opsonin-induced phagocytic destruction of target cells by macrophages studied by Old, Boyse, Bennett, and Lilly (*10*).

That adherence, the first step in the interaction of the immune cell and the target cell, is specific leads us to favor the concept that this may be the only specific component in the interaction and that by inducing intimate contact of cell membranes it may promote exchange of cytoplasmic materials which secondarily leads to cell injury.

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Fetal Death from Nicotinamide-Deficient Diet and Its Prevention by Chlorpromazine and Imipramine

Abstract. *Feeding a diet deficient in nicotinamide, nicotinic acid, and tryptophan to pregnant rats causes death and resorption of all fetuses. This effect can be prevented by administration of either chlorpromazine or imipramine. Analysis of maternal liver at the time of fetal resorption indicates that the observed effects may be mediated through modification of the concentration of the pyridine nucleotide coenzymes.*

Fetal abnormalities, fetal death, and fetal resorption have been produced by diets deficient in any one of several different vitamins (*1*). However, the effect on fetal development of feeding the mothers diets deficient in nicotinamide does not appear to have been reported, although 6-aminonicotinamide, a nicotinamide antagonist, has been shown to be teratogenic (*2*).

We have now found that feeding pregnant rats a diet deficient in all three known sources of nicotinamide—nicotinamide, nicotinic acid, and tryptophan—from the first day after conception, resulted in the loss of fetal viability (Table 1).

The increase in the concentration of nicotinamide adenine dinucleotide (NAD) which occurs in response to administration of nicotinamide, is maintained for a longer period of time if animals are treated first with either of the tranquilizers chlorpromazine or reserpine (*3*). Since the mechanism by which the tranquilizers cause the increase of NAD to be maintained is being investigated in this laboratory (*4, 5*), it was of interest to determine whether chlorpromazine would manifest a "nico-