Interferon Production by Macrophages from Adult and Newborn Rabbits Bearing Fibroma Virus-Induced Tumors

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Tumors were induced in adult and newborn rabbits by inoculation of fibroma virus. After 10 to 14 days, oil-induced peritoneal macrophages were harvested, purified, and tested in vitro for interferon synthesis after stimulation with specific and nonspecific viruses. Peritoneal macrophages from adult rabbits that had initiated tumor regression produced high levels of interferon (titers ranged from 160 to 640) after stimulation with fibroma virus, whereas macrophages from normal adult rabbits failed to produce significant levels of interferon under the same conditions (titers ranged from <10 to 10). Furthermore, fibroma-immune macrophages responded to vaccinia virus and Newcastle disease virus with higher levels of interferon than did normal macrophages. In contrast, macrophages from newborn tumor-bearing rabbits that showed no evidence of tumor regression failed to respond to fibroma virus stimulation with higher levels of interferon (titers ranged from <10 to 10). These macrophages did, however, yield significantly more interferon than newborn control macrophages when stimulated with a good interferon inducer, Newcastle disease virus (titers ranged from 10 to 80). These data suggest that interferon production may be an expression of macrophage activation to fibroma antigens and that macrophage activation is impaired in newborn rabbits with progressive growing tumors.

Recent studies in this laboratory have been concerned with the role of the immune response in regression of tumors induced in adult rabbits by Shope fibroma virus and the apparent failure of the hosts' immunity in newborn rabbits that frequently support progressive tumor growth.

Humoral antibody response to Shope fibroma virus and virus-induced tumor has been reported to be similar in newborn and adult rabbits as judged by virus neutralization (1) and cell cytotoxicity tests (15). Although adult tumor-bearing rabbits demonstrated strong cell-mediated immunity to fibroma antigens as measured by the delayed cutaneous hypersensitivity reaction, passive lymphocyte transfer reaction, and the macrophage migration inhibition test, newborn rabbits with progressively growing tumors showed markedly impaired lymphocyte reactions (1, 16). Thymus-dependent lymphocytes, sensitized to a variety of antigens, are known to produce a number of factors on re-exposure to the specific antigen that mediate immunological reactions. Some of these mediators apparently act upon and activate macrophages. There is increasing evidence that such activated macrophages may play a prominent role as effector cells in tumor immunity (7, 8; R. T. Osteen and W. H. Churchill, Fed. Proc. 31:610, 1972).

In the case of regression of fibroma-induced tumors, macrophages might function by direct interaction with and destruction of tumor cells, by destruction of phagocytosed virus, or by inhibition of virus replication in susceptible cells through interferon production. Qualitative or quantitative failure in one or all of these possible functions could result in progressive tumor growth in newborn rabbits.

Prose et al. (13) demonstrated that increased interferon titers preceded the decrease in infective virus titer in fibroma tumors and suggested that interferon contributes to resolution of the lesion. Furthermore, Smith et al. (13) showed that extracts of tumors from adult rabbits contained significantly higher levels of interferon than similar extracts from newborn rabbits. It is conceivable that the difference in interferon levels in tumors from adult and newborn rabbits may reflect the difference in infiltrating mononuclear cells.

Histological studies have demonstrated that regression of fibroma tumors in adult rabbits is preceded by infiltration and formation of a dense ring of mononuclear cells around the
tumors. However, tumors induced in newborn rabbits grow progressively and demonstrate only minimal mononuclear cell infiltration (1). This study was designed to investigate interferon production by peritoneal macrophages from adult and newborn tumor-bearing rabbits.

MATERIALS AND METHODS

Viruses and virus assay. The Patuxent strain of Shope fibroma virus and the WR strain of vaccinia virus were grown and assayed as described previously (16). Newcastle disease virus (NDV) was grown on primary chicken embryo fibroblasts in 16-oz (453.6-g) prescription bottles, harvested at 48 h, and assayed on the same cells under a 1% methyl cellulose overlay for plaque-forming units (PFU). The Indiana strain of vesicular stomatitis virus (HSV) was grown on primary rabbit kidney (PRK) cells and assayed for PFU on the same cells under a 1% agar overlay. All virus stocks were stored at -70 C in 1-ml amounts.

Cell cultures. PRK cells were prepared by overnight trypsinization of kidneys from 3- to 4-week-old New Zealand white rabbits. The dispersed cells were sedimented, washed twice with Hanks balanced salt solution (HBSS), and suspended to a concentration of 5 × 10⁶ cells/ml in medium 199 containing 10% fetal calf serum (FCS), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.075% sodium bicarbonate. Cells were seeded in 25-ml quantities in 16-oz prescription bottles or in 4-ml quantities in 60-mm plastic petri plates. The cells formed a confluent monolayer by days 5 to 6 with a change in medium on day 3.

Primary chicken embryo fibroblasts cultures were prepared from 10-day-old chicken embryos. After decapitation and removal of wings and legs, the rest of the embryos were trypsinized with 0.25% trypsin and dispersed cells were collected every 20 min. The dispersed cells were washed twice with HBSS and suspended in medium 199 containing 10% trypsinase phosphate broth in addition to 5% FCS, streptomycin, penicillin, and sodium bicarbonate. The suspension was filtered through a double layer of cheesecloth, adjusted to a concentration of 5 × 10⁶ cells/ml, and seeded in 25-ml quantities in 16-oz bottles. The confluent monolayers were formed by 24 h of incubation.

DRK₃ cells, an established line of rabbit kidney epithelial cells, were grown as described earlier (15).

Immunization of rabbits. Tumors were induced in young adult rabbits, 3 to 4 months old, by intracutaneous inoculation with 2.5 × 10⁶ PFU of fibroma virus at each of six sites on the back. Newborn rabbits were inoculated on day 1 or 2 of age with the same amount of virus subcutaneously.

Fibroma virus-induced tumors in adult and newborn rabbits have been described in detail in earlier publications (16).

All experiments were performed with macrophages collected from rabbits at 10 to 14 days after virus inoculation. Preparation of peritoneal macrophages. Peritoneal exudate cells were prepared as described by Tompkins et al. (16). Although adult rabbits yielded markedly more cells in the peritoneal exudates than newborn rabbits, the suspensions consisted of 80 to 90% macrophages in both cases. (In studies with newborns, macrophages were pooled from several animals for each experiment.) The peritoneal exudates were suspended in medium 199 to a concentration of 2.5 × 10⁶ cells/ml. A 3-ml amount of the cell suspension was seeded in each of 1-oz (28.35-g) glass prescription bottles and incubated at 37 C for 30 min. Nonadhering and loosely adhering cells were removed by washing twice with HBSS and gentle shaking. No significant difference was observed in the number of adhering cells in adult and newborn preparations.

Interferon production. Macrophage cultures were inoculated in duplicate with ultraviolet (UV)-irradiated vaccinia virus (1 PFU/cell), fibroma virus (10 PFU/cell), and NDV (10 PFU/cell). UV irradiation of the virus was performed by exposing a thin layer of virus suspension to a UV lamp at a distance of 5 cm for 3 min with rotation of the suspension every 30 s. After 2 h of virus adsorption at 37 C, unadsorbed virus was removed by washing, 3 ml of medium 199 was added, and the cultures were incubated at 37 C for 24 h. In each experiment, uninfected cultures in duplicate were included. The medium was then collected, acidified with 1 N HCl to pH below 3.0, and refrigerated for 24 h to destroy the virus. The interferon preparations were neutralized with 1 N NaOH, diluted to 1:10 with medium 199 containing 2% FCS, and centrifuged at 30,000 rpm for 1 h in a Beckman model L350 ultracentrifuge with a T-60 rotor. The supernatant was collected and stored at -70 C in 10-ml portions until assayed.

Interferon assay. Interferon was assayed by addition of 2 ml of serial twofold dilutions (medium 199 plus 2% FCS) in duplicate to PRK monolayers in 60-mm plastic petri plates. After 18 to 20 h of incubation at 37 C, the medium was removed and the monolayers were infected with 50 to 100 PFU of VSV. After 2 h of virus adsorption, the cells were overlayed with a 1% agar overlay medium and further incubated to 48 h at 37 C. The number of plaques was recorded after fixing the monolayers with formaldehyde (2 parts) ethyl alcohol (20 parts), and glacial acetic acid (1 part) and staining with crystal violet. The interferon titer was recorded as the reciprocal of the dilution that reduced the number of plaques by 50%.

RESULTS

Production of interferon by macrophages for normal and tumor-bearing adult rabbits. Purified peritoneal macrophages from normal adult rabbits produced low levels of interferon (titers ranged from <10 to 10) when stimulated by fibroma virus or vaccinia virus in culture. For the most part, these values corresponded to interferon titers for cultures of unstimulated macrophages (Table 1). Normal macrophages stimulated by NDV produced interferon titers of 40 in two experiments.

In contrast, macrophages from adult fibroma tumor-bearing rabbits (10 to 14 days after virus inoculation) produced high titers of interferon...
Table 1. Interferon production by cultured peritoneal macrophages from normal and fibroma tumor-bearing adult rabbits

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Rabbits* immunized against</th>
<th>Macrophage interferon titer on stimulation by*</th>
<th>Un-</th>
<th>Fibroma virus</th>
<th>Vaccinia virus</th>
<th>NDV</th>
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<td>&lt;10</td>
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<tr>
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<tr>
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<td>160</td>
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*Peritoneal macrophages harvested 10 to 14 days after tumor induction by intradermal inoculation of fibroma virus.

*Interferon collected 24 h after inoculation of monolayer cultures of 7.5 x 10^6 macrophages with UV-irradiated virus. Interferon titer is expressed as the reciprocal of the dilution that reduced the number of VSV plaques by 50%.

*NT, Not tested.

levels of interferon after virus stimulation (Table 2). Furthermore, macrophages from newborn tumor-bearing rabbits behaved as normal macrophages in that they failed to produce significant levels of interferon after stimulation by fibroma virus or vaccinia virus. Interferon titers from these rabbits were 10 or less in all experiments as compared with <10 for normal macrophages. In the case of NDV stimulation, interferon titers from tumor-bearing rabbits ranged from 10 to 80 as compared with 10 from normal rabbits.

It is clear from these experiments that, although the interferon response to specific and nonspecific inducers is markedly enhanced in macrophages from adult tumor-bearing rabbits, macrophages from newborn tumor-bearing rabbits show only minimal activation with respect to interferon production.

DISCUSSION

The results in these experiments indicate that peritoneal macrophages from fibroma tumor-bearing adult rabbits have increased ability to produce interferon in response to a viral stimul. Such enhanced interferon production by immune macrophages has been reported by Glasgow (10) and Yamada et al. (18). However, Glasgow (10) and Azuma et al. (3) reported that increased ability of macrophages to produce interferon is specific for the immunizing virus, which is contrary to the results presented here.

Table 2. Interferon production by cultured peritoneal macrophages from normal and fibroma tumor-bearing newborn rabbits

<table>
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<th>Rabbits* immunized against</th>
<th>Macrophage interferon titer on stimulation by*</th>
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<td>&lt;10</td>
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</tbody>
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*Peritoneal macrophages harvested 10 to 14 days after tumor induction by subcutaneous inoculation of fibroma virus.

*Interferon collected 24 h after inoculation of monolayer cultures of 7.5 x 10^6 macrophages with UV-irradiated virus. Interferon titer is expressed as the reciprocal of the dilution that reduced the number of VSV plaques by 50%.

*NT, Not tested.
Our studies indicate that within the systems studied, specificity was not involved since fibroma-immune macrophages produced higher levels of interferon on stimulation by vaccinia virus and NDV as well as fibroma virus.

Whether increased interferon production by immune macrophages is due to the increased adsorption of the virus is not certain. Whereas Glasgow (10) found no increased adsorption of the specific virus to the immune macrophages, Azuma et al. (3) reported increased adsorption. Previous studies in this laboratory demonstrated no significant difference in adsorption of vaccinia virus (2) or fibroma virus (16) to immune and normal macrophages.

It is unlikely that the increased interferon production by immune macrophage cultures was due to lymphocytes since most of the lymphocytes were removed from the culture by washing the monolayers prior to stimulation for interferon production. Therefore, from our data it is likely that increased interferon production represents an intrinsic characteristic of activated macrophages in much the same manner as previously reported increased bactericidal (4), virucidal (2, 10), and tumoricidal (9, 11) activity after activation. However, this remains only a conjecture, because the mechanisms of interferon synthesis as well as macrophage activation are still uncertain.

Although macrophages from newborn tumor-bearing rabbits failed to respond to stimulation by fibroma virus in two experiments, they did yield slightly higher levels of interferon than normal macrophages when stimulated with NDV. This datum could be interpreted to suggest that interferon production is a result of macrophage activation to the fibroma tumor antigens and that macrophage activation is depressed in newborn rabbits to such an extent that the cells respond only to a strong interferon inducer, such as NDV. In support of this, it has been suggested that the generalized fibromatosis sometimes seen in newborn rabbits infected with fibroma virus might be due to the relative immaturity of the reticuloendothelial barriers to the spread of virus (6).

In the present study we obtained low yields of interferon as compared with previous studies using mouse and rabbit macrophages (10, 14). This was particularly evident in the case of normal macrophages that produced very little interferon even when stimulated by NDV. The most plausible explanation for these differences is that previous experiments utilized 10- to 100-fold more macrophages than were used in these experiments. It was necessary to use small numbers of macrophages in these studies since newborn rabbits yield few exudate cells after intraperitoneal injection of mineral oil.

The potential role of macrophage interferon on fibroma tumor regression has not been determined. Prose et al. (12) demonstrated interferon in fibroma tumors preceding regression and suggested it may be a factor in the tumor regression. Similarly, Vilcek et al. (17) demonstrated that treatment of rabbits with poly(I:C) resulted in interferon production and suppression of fibroma tumors.

Interferon might restrict the size of fibroma tumors by reducing the susceptibility of surrounding normal tissue and preventing spread of the virus. Chan et al. (5) demonstrated that fibroma virus was sensitive to poly(I:C)-induced interferon in PRK cells. However, Smith et al. (13) found no correlation between interferon titer and tumor regression. They demonstrated maximal titers at 2 days after virus inoculation and very little interferon after 4 days. The tumors increased significantly in size, and maximal virus titers in the tumors were achieved after this time. This was similar in both adult and newborn rabbits with the exception that lower titers of interferon were found in newborn tumors. This corresponds with our observation that macrophages from newborn tumor-bearing rabbits do not produce large amounts of interferon after stimulation by fibroma antigens. At this time the role of interferon in fibroma tumor regression remains unresolved.

Failure of macrophages from newborn tumor-bearing rabbits to respond to viral interferon inducers raises the possibility that newborn rabbits have an impaired macrophage activation mechanism. Previous studies by Allison (1) and Tompkins et al. (16) presented evidence for a reduced thymus-dependent lymphocyte function in newborn tumor-bearing rabbits. Because macrophage activation is considered by many to be mediated by a factor(s) released from specifically stimulated lymphocytes (7–9), it follows that reduced lymphocyte function might result in an impairment of macrophage activation. Experiments are now in progress to further investigate the immunological activity of macrophages from newborn and adult tumor-bearing rabbits and the interaction between thymus-dependent lymphocytes and macrophages in these systems.

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MACROPHAGE INTERFERON PRODUCTION

LITERATURE CITED