Demonstration of Cytotoxic Antibodies in Rabbits Bearing Tumors Induced by Shope Fibroma Virus

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The $^{51}$Cr-release test was used to detect cytotoxic antibodies in adult rabbits bearing tumors induced by Shope fibroma virus. The following are the recommended experimental conditions: the infection of RK-13 cells with a multiplicity of 1 to 2 infectious units per cell for 48 hr, $^{51}$Cr labeling of infected cells during the last 12 hr of incubation, sensitization of suspended labeled infected cells for 1 hr with immune serum, and quantitation of cell damage by the amount of $^{51}$Cr released after 6 hr of incubation in the presence of complement. The immune sera reacted only with fibroma virus-infected cells but not with cells infected with vaccinia virus or herpesvirus type 1. Similarly, sera prepared against vaccinia virus and herpesvirus type 1 were not cytotoxic to fibroma virus-infected cells, although they were cytotoxic to cells infected with homologous viruses. The total antibody activity in sera of rabbits infected with Shope fibroma virus was detected first on day 7, gradually rose to its peak by day 23, and persisted at that level for at least 50 days. The 19S antibody was detected on day 7, reached peak titers by day 13, and disappeared by day 17. The 7S antibody was barely detectable on day 7, reached maximum titers on day 13, and remained high for at least 50 days. The tumors appeared on the 3rd day after virus inoculation, reached maximum size on day 13, and regressed completely by day 23.

In adult rabbits, Shope fibroma virus induces localized benign tumors which regress spontaneously. The rabbits bearing tumors develop a cell-mediated immune response, as measured by the delayed-type hypersensitivity reaction (1, 2, 13, 21) and macrophage migration-inhibition test (21, 22). In addition, they develop a humoral immune response as shown by the development of virus-neutralizing antibodies (1, 2, 4, 6, 13) and antibodies which react with antigens of fibroma virus-infected cells in the immunofluorescence test (16, 22). The surface antigens, either virion or new antigens specified by the virus, in cells transformed by oncogenic viruses play a major role in the development of tumor immunity (9, 14). The humoral antibodies against antigens at the surface of virus-free tumor cells have been implicated in the enhancement of tumor growth rather than in regression (11). However, when virion antigens are present at the surface of tumor cells, antibodies develop which can bring about in vitro complement-mediated cytolysis of the tumor cells (17).

This study was undertaken to determine whether complement-dependent cytotoxic antibodies were synthesized by rabbits bearing tumors induced by Shope fibroma virus and to study the kinetics of cytotoxic antibody response in relation to the development and regression of tumors. Attempts were also made to characterize the cytotoxic antibodies with respect to the immunoglobulin class.

MATERIALS AND METHODS

Cell cultures and media. Primary rabbit kidney (PRK) cells were prepared from 3-week-old New Zealand White rabbits (15). The cells were grown and maintained in Eagle’s minimal essential medium containing 10% heat-inactivated fetal calf serum (FCS), 100 units of penicillin, and 100 $\mu$g of streptomycin per ml. A continuous cell line of rabbit kidney cells, RK-13 (3), was grown in medium 199 supplemented with 10% heat-inactivated FCS and antibiotics.

Viruses. The Patuxent strain of Shope rabbit fibroma virus, the KOS strain of herpesvirus type 1, and the WR strain of vaccinia virus were prepared in PRK cells as described earlier (22). Herpesvirus type 1 and vaccinia virus were titrated by the plaque method in PRK cells (18). Fibroma virus was assayed in RK-13 cells by modification of a focus formation method previously described (1). Briefly, 10-fold serial dilutions of fibroma virus in 0.2-ml volumes were allowed to adsorb onto cell monolayers in 60-mm diameter petri dishes (Falcon Plastics) at 37 C. After 2 hr, 5 ml of an overlay consisting of 0.3% agarose in growth medium was added to each plate. After 7 days
of incubation at 37 C in a 5% CO2 humidified atmosphere, the cell sheet was stained with 0.5% crystal violet, and foci of virus infection were counted. Titers were expressed as focus-forming units (FFU) per milliliter. The stock virus had a titer of 10^8.8 to 10^9 FFU per ml.

**Inoculation of rabbits with fibroma virus.** Five adult New Zealand White rabbits were inoculated intradermally with 0.1 ml of fibroma virus (10^6.8 to 10^7.8 FFU) at each of six well-separated locations on their shaved backs. The animals were examined at frequent intervals for development of tumors. The measurements of the lesions were made with the help of Vernier calipers. The fibroma virus-infected rabbits were bled by ear vein puncture at various time intervals during tumor development and regression, and the sera, after heat inactivation at 56 C for 30 min, were frozen at -20 C without preservatives until assayed for cytotoxic activity by the 51Cr-release test.

**Chromatographic separation of immunoglobulins.** The immune sera in 5-ml amounts were fractionated by gel filtration on Sephadex G-200 columns (2.5 by 75 cm) equilibrated with 0.1 M borate buffer, pH 8.2 (7). Fractions were collected in 5-ml amounts by use of an automatic fraction collector and were analyzed for protein content by measuring absorbance at 280 nm in a Beckman DU spectrophotometer. Preliminary tests showed that cytotoxic antibody activity was present only in fractions constituting peak I and peak II. Therefore, fractions under peak I (19S) and peak II (7S) regions were pooled separately, concentrated to 5-ml volumes, and dialyzed against normal saline. The 19S and 7S nature of immunoglobulins obtained after Sephadex G-200 chromatography was further investigated by treating the immunoglobulins with 0.1 M 2-mercaptoethanol at room temperature for 4 hr followed by dialysis for 12 hr against large volumes of normal saline containing 0.02 M iodoacetamide (19). Untreated and 2-mercaptoethanol-treated fractions were then assayed for cytotoxic activity against fibroma-infected cells.

**Assay for cytotoxic antibody by 51Cr release.** Cytotoxic antibody was detected by use of a modification of Goodman’s method (8). Cell monolayers (RK-13), in 8- to 16-oz (ca. 240- to 470-ml) prescription bottles, were infected with fibroma virus at a multiplicity of infection (MOI) of approximately 2 FFU/cell. After 36 hr of incubation, 200 μCi of 51Cr (as sodium chromate, Hastings Radiochemical Works, Inc.) was added to the cultures which were further incubated for an additional 12 hr. The labeled cells were dispersed by use of a mixture of equal parts of trypsin (0.25%) and ethylenediaminetetraacetate (0.02%), and were washed five times with cold tris-(hydroxymethyl)aminomethane-buffered saline (TBS) supplemented with 10% heat-inactivated FCS. To 0.1-ml amounts of labeled cell suspension (5 × 10^4 cells) in Falcon plastic culture tubes (16 by 125 mm) was added an equal volume of an appropriate dilution of test serum. After incubation at 37 C for 1 hr, 0.2 ml of a 1:2 dilution of guinea pig complement was added, and incubation was continued for an additional 6 hr. After the addition of 2 ml of cold TBS with FCS to each tube, the cells were sedimented at 1,500 rev/min for 10 min at 4 C. A 1-ml amount of the supernatant fluid was then transferred to another tube. The tubes were counted for radioactivity in a Nuclear-Chicago automatic gamma spectrometer. The counts obtained from 1 ml of supernatant fluid were multiplied by 2.4 (to adjust for the volume left with the sediment) to obtain total radioactivity released. Controls consisted of: uninfected labeled cells, preimmune serum in place of immune serum, heated complement in place of unheated complement, TBS with FCS in place of complement and immune serum, no complement, and no immune serum.

The cytotoxic activity of the test serum was expressed as per cent specific 51Cr release, which was calculated by use of the following formula:

Per cent specific 51Cr release equals:

\[
\text{per cent specific } 51\text{Cr release} = \frac{\text{maximum release} - \text{spontaneous release}}{\text{maximum release}} \times 100
\]

The maximum release was taken as the amount of radioactivity released by freezing and thawing the prelabeled target cells once in the presence of water. Ab and C represent antibody and complement, respectively.

**RESULTS**

**Measurement of cytotoxic antibodies.** Initially, RK-13 cells were infected with Shope fibroma virus and harvested 48 hr after infection, a time previously shown to be required for maximum appearance of surface antigens in fibroma virus-infected cells (21). The labeled cells were mixed with undiluted immune serum from rabbits whose fibromas regressed. After 1 hr of incubation, un-

![Fig. 1. Kinetics of 51Cr release from infected cells in the presence of complement. To the sensitized pre-labeled fibroma virus-infected cells, complement was added, replicate cultures were harvested at various time intervals, and the radioactivity released was measured. Each point represents the difference in counts obtained in the presence of active and heat-inactivated comple-ment.](http://iai.asm.org/ http://iai.asm.org/ on August 14, 2017 by guest)
diluted guinea pig serum was added as a source of complement. Replicate tubes were then harvested at various time intervals to measure the kinetics of release of $^{51}$Cr from the infected cells. The results in Fig. 1 show that there was a rapid increase in the release of $^{51}$Cr within the first 2 hr of reaction, followed by a gradual increase in the specific release of $^{51}$Cr. In all subsequent experiments, the radioactivity was harvested 6 hr after the addition of complement.

The optimal dilution of complement for use in the cytotoxicity test was determined by mixing serial twofold dilutions of guinea pig serum with $^{51}$Cr-labeled target cells (Fig. 2). Maximum specific $^{51}$Cr release was obtained when undiluted guinea pig serum was used. The $^{51}$Cr release was close to maximal at a 1:2 complement dilution and then decreased linearly with increasing dilutions of the complement. On the basis of these results, a 1:2 dilution of guinea pig serum was chosen as a source of complement for subsequent experiments.

The results of a typical cytotoxicity assay by $^{51}$Cr release with fibroma virus-infected and uninfected cells and antisera are presented in Table 1. There was 75% specific release of radioactivity from infected cells in the presence of immune serum and complement. There was no significant difference among the counts released from infected cells by (i) immune serum and heated complement, (ii) normal serum and active complement, and (iii) medium alone. The counts released after freezing and thawing the labeled cells represented the maximum release.

**Kinetics of antigen appearance after virus infection.** Experiments were conducted to examine the synthesis of antigen which reacts with cytotoxic antibody. RK-13 cells were infected with a 1 to 2 MOI of fibroma virus. At various time intervals after infection, the cells were labeled with $^{51}$Cr and, after dispersion into single-cell suspension, were used as target cells in the cytotoxicity assay using antibody from rabbits whose fibromas had regressed and 1:2 dilution of guinea pig complement. The results presented in Fig. 3 show that

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**TABLE 1. Demonstration of complement-dependent cytotoxic antibodies by $^{51}$Cr-release test**

<table>
<thead>
<tr>
<th>$^{51}$Cr release in the presence of</th>
<th>Fibroma virus-infected cells</th>
<th>Uninfected cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Counts per min$^a$</td>
<td>Per cent release</td>
</tr>
<tr>
<td></td>
<td>Total incorporation Released</td>
<td>66.0</td>
</tr>
<tr>
<td>Immune serum$^b$ + complement$^c$</td>
<td>2,270 1,501</td>
<td></td>
</tr>
<tr>
<td>Immune serum + heat-inactivated complement$^d$</td>
<td>2,065 367</td>
<td>17.8</td>
</tr>
<tr>
<td>Preimmune serum + complement$^e$</td>
<td>2,116 408</td>
<td>19.3</td>
</tr>
<tr>
<td>Medium (spontaneous)</td>
<td>2,127 352</td>
<td>16.5</td>
</tr>
</tbody>
</table>

$^a$ Average of two tubes.
$^b$ Immune serum from rabbit 543 collected 50 days after infection with Shope fibroma virus. Serum was treated at 56°C for 30 min.
$^c$ Guinea pig serum diluted 1:2 as source of complement.
$^d$ Heat inactivation was done at 56°C for 30 min.

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here was no specific release of radioactivity from cells infected for 12 hr. Thereafter, there was a linear increase in the release of radioactivity which reached a maximum at 48 hr and remained nearly at that level for 72 to 96 hr of infection.

Effect of MOI. RK-13 cells growing in 8-oz prescription bottles were infected with MOI of 2, 0.2, 0.02, and 0.002 of fibroma virus. The infected cells after 48 hr were used as target cells in the cytotoxic assay. The results presented in Fig. 4 show that the specific release of radioactivity from the infected cells by antibody in the presence of complement was dependent upon the virus concentration used to infect the cells. Maximum specific release was obtained with maximum virus input.

Specificity of reaction. The immunological specificity of reaction to fibroma virus was investigated by reacting immune serum from fibroma-infected rabbits against cells infected with vaccinia and type 1 herpes simplex viruses in the 51Cr-release assay. Also, fibroma virus-infected cells were reacted with antisera to vaccinia, herpes simplex type 1, and Shope papilloma viruses. Results presented in Table 2 clearly show that only antiserum from fibroma-infected rabbits was effective in bringing about cytolysis of fibroma virus-infected cells, whereas antisera to type 1 herpes simplex virus, vaccinia virus, and Shope papilloma virus had no effect. The specificity of the reaction was strengthened by the observations that immune serum from fibroma-infected rabbits did not bring about complement-dependent cytolysis of cells infected with either vaccinia virus or herpes simplex virus, as these infected cells were lysed only by immune sera directed against the respective viruses. These studies show that the immune response is specifically directed to antigen(s) specified by the fibroma virus.

The specificity and the quantitative nature of antigen at the surface of fibroma virus-infected cells was further suggested by results of adsorption studies of cytotoxic activity of fibroma immune serum by unlabeled fibroma-infected cells (Fig. 5). Increasing numbers of unlabeled fibroma virus-infected or uninfected cells were incubated with 0.1 ml of 1:10 dilution of fibroma-immune serum for 1 hr, at which time $5 \times 10^4$ labeled fibroma virus-infected cells were added to each tube. After further incubation for 1 hr at 37°C, 0.2 ml of a 1:2 dilution of guinea pig serum was added as a source of complement. The radioactivity released in the supernatant fluid was measured after 6 hr. The results show that, by using an increasing number of fibroma virus-infected cells, it was possible to progressively absorb out the cytotoxic activity of immune serum. Uninfected cells failed to absorb the antibody.

Quantitative determination of cytotoxic antibody. Immune sera from two rabbits (no. 543 and 653) collected 50 days after infection with fibroma virus were serially diluted and were tested for complement-dependent cytotoxicity by use of 51Cr-labeled fibroma virus-infected cells. The results presented in Fig. 6 show that cytotoxic activity could be detected in the sera from both of the rabbits at a 640-fold dilution. The maximum specific release was obtained with antiserum dilu-
The sera of rabbits infected with fibroma virus, vaccinia virus, or HSV type 1 were assayed for cytotoxic antibodies. The sera were tested for specific cytotoxicity against fibroma virus-infected cells by the Cr-release assay. Also, immune sera collected on days 7, 13, 17, and 50 after infection were subjected to Sephadex G-200 chromatography. The fractions under 19S and 7S peaks were pooled, concentrated to the same volume as that of the antiserum, and tested for cytotoxic antibodies. The sensitivity of 19S and 7S material to 2-mercaptoethanol was also tested. The results presented in Fig. 7 show that cytotoxic antibody in the sera of rabbits could be demonstrated 7 days after infection with fibroma virus and reached a maximum level on day 23, remaining constant thereafter for at least 50 days. The 7S antibody response was maximal on day 13 and then remained parallel to the total antibody response. The cytotoxic activity of 7S fractions did not decrease significantly after 2-mercaptoethanol treatment. The 19S antibodies could be detected in sera collected on the 7th day postinfection. They reached a maximum on day 13 and were barely detectable in sera collected on the 17th day. No cytotoxic activity could be demonstrated in 2-mercaptoethanol-treated 19S preparations. Tumors could be detected by the 3rd day after virus inoculation with fibroma virus. See Fig. 7 for details.

Fig. 5. Absorption of cytotoxic antibody activity by various concentrations of fibroma virus-infected cells.

Fig. 6. Quantitation of cytotoxic antibody activity of day 50 fibroma-immune sera from rabbits.

TABLE 2. Immunological specificity of cytotoxic antibody to fibroma virus-infected cells

<table>
<thead>
<tr>
<th>Cells infected with</th>
<th>Per cent specific release in the presence of immune seruma + complement</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Rabbit anti-fibroma</td>
</tr>
<tr>
<td>Fibroma virusd</td>
<td>72</td>
</tr>
<tr>
<td>Vaccinia virusb</td>
<td>1</td>
</tr>
<tr>
<td>HSV type 1</td>
<td>12</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
</tbody>
</table>

a All sera were diluted 1:4 and treated at 56°C for 30 min before use.
b Kindly supplied by J. S. Butel.
c The human serum contained neutralizing antibodies to both vaccinia virus and herpesvirus.
d RK-13 cells were infected with fibroma virus at a MOI of approximately 2 and incubated for 48 hr. Cells were infected with vaccinia virus or herpesvirus (HSV) at a similar multiplicity and incubated for 18 hr.
e Not done.
inoculation, and they reached maximum size on day 13. The tumors had regressed completely by the 23rd day.

**DISCUSSION**

In this study, the presence of cytotoxic antibodies in rabbits bearing tumors induced by the inoculation of Shope fibroma virus has been demonstrated, by use of the $^{51}$Cr-release method. The $^{51}$Cr release assay is accurate, sensitive, and highly reproducible, and there is no reutilization of released $^{51}$Cr by the target cells (5, 20, 23). $^{51}$Cr release by antibody has also been demonstrated in other virus-antibody systems (10, 12; A. M. Brier, C. R. Wohlenberg, and A. L. Notkins, Fed. Proc. 30:906, 1971). The assay system was standardized in terms of cultural and experimental conditions to obtain optimal specific release of $^{51}$Cr from the target cells. The spontaneous release of radioactivity was found to be very low under the experimental conditions, and therefore low levels of immune lysis could be measured.

The immune reaction between cytotoxic antibodies and antigen(s) at the surface of fibroma virus-infected cells was specific, since the fibroma postinfection sera failed to react with cells infected with either vaccinia or herpes simplex virus. Similarly, antisera to herpes simplex, vaccinia, and Shope papilloma viruses did not react with fibroma virus-infected cells in the $^{51}$Cr-release test. Moreover, the cytotoxic activity of the immune sera could be absorbed with fibroma virus-infected cells but not with uninfected cells, ruling out the immune reaction against histocompatibility antigens.

Although cellular immunity appears to play a role in the regression of the lesions induced by fibroma virus (1, 2), the results presented here suggest that humoral immune response may also take part in the regression of the lesions. The host develops cytotoxic antibodies and the kinetics of the humoral immune response parallel tumor development and regression. Rabbits developed visible tumors on the 3rd day after virus inoculation. The tumors continued to grow until 12 to 13 days, after which time the tumors started to regress. The regression of tumors was complete by the end of the 3rd week. The total antibody response was demonstrable on the 7th day, reaching a maximum by the end of the 3rd week and remaining constant thereafter. The $19S$ antibody response was demonstrable on the 7th day, peaked on day 13, and dropped to a low level by the 17th day, whereas the $7S$ antibody response paralleled the total antibody response. These data suggest that both $19S$ and $7S$ antibodies may be participating in the regression of tumors.

The specific antigens at the surface of cells infected with fibroma virus had been previously demonstrated (16, 22). Our findings are in agreement with those of Tompkins et al. (22) on the kinetics of appearance of antigens measured by indirect membrane fluorescence test. At this writing, there is no evidence as to whether the antigens reacting in the cytotoxicity test and the immunofluorescence test are the same or different. It is also not clear whether these antigens are virion antigens or virus-specified antigens. The demonstration of neutralizing antibodies in tumor-bearing rabbits (1) indicates the presence of virion
antigens at the tumor cell surface. Whether neutralizing antibodies are cytotoxic is not known at this time. The role of virion antigens at the tumor cell surface in inducing an immune response, either cellular or humoral, to bring about tumor regression is also not clear. Tumors induced by fibroma virus in newborn rabbits do not regress, but the animals develop neutralizing antibodies (1, 6). Experiments are in progress to test whether rabbits inoculated with fibroma virus as newborns will develop cytotoxic antibodies. These should shed some light on the role of cytotoxic antibodies in tumor regression.

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LITERATURE CITED