The Effect of Virus-immune Serum on Anti-viral Cytotoxic T cells in vivo and in vitro

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SUMMARY

Virus-immune sera applied to infected target cells inhibited Tc cell-mediated lysis in vitro. Blocking activity was clearly present in serum by 6 days after ectromelia virus infection. Activity was found in the IgG and IgM fractions of hyper-immune sera and was specific for the immunizing virus when ectromelia and influenza viruses were used, but did not distinguish between the serologically related poxviruses ectromelia, vaccinia and rabbitpox. There was no requirement for the donors of immune serum to be of the same mouse strain as the target cells, or the same species, since rabbit sera blocked similarly to mouse sera.

These findings imply that virus-specified antigens recognized by B cells are physically close to, or identical to, the virus antigens involved in Tc cell-recognizable antigenic changes in infected cell surfaces. There was no evidence for modified H-2 molecules alone, or other proteins coded by derepressed host cell genes being recognized by virus-immune Tc cells. Significant inhibition of lysis by anti-viral antibody was only observed on fibroblast type target cells. Macrophage and P815 targets were refractory to blocking. These findings are discussed in practical terms and in relation to possible regulation of Tc cell responses in vivo by anti-viral antibody.

INTRODUCTION

Virus infection of murine cells leads to the expression of virus-coded and/or virus-induced antigens on the cell surface. Virus-immune cytotoxic T cells (Tc cells) recognize and lyse infected cells, but display dual specificity for the virus and for the H-2K and/or H-2D antigens of the murine host (Doherty et al. 1976). Two different mechanisms can produce the antigenic patterns recognized by Tc cells. Virion envelope proteins can be transferred into the cell surface membrane by fusion of the virus envelope with the cell membrane. This mechanism occurs most efficiently with paramyxoviruses (Schrader & Edelman, 1977), but with a high m.o.i. it can also occur with other virus groups (Hapel et al. 1978). With lower m.o.i., new protein synthesis early after penetration and uncoating is required for antigenic changes detectable by Tc cells (Ada et al. 1976; Jackson et al. 1976). Proteins specified either by the virus genome, or by host cell genes derepressed after infection could produce the relevant antigenic changes.

Models to account for Tc cell recognition fall into two general categories, dual receptor or single receptor (Blanden & Ada, 1978; Langman, 1978). Dual receptor models invoke two separate Tc recognition sites for H-2 and virus-induced antigens, though available evidence suggests that to trigger Tc cell function these sites must act as a paired set to recognize two antigen molecules close together in the same cell membrane. Single receptor models envisage one class of recognition site that binds to a modified self H-2 molecule, or
to a new antigenic determinant resulting from a complex of self H-2 and virus-induced molecules. It is apparent that sub-classes of each model can be proposed, depending upon whether the new virus-induced protein(s) are coded by the virus genome or the host cell genome. Theoretically these sub-models can be tested if Tc cell recognition and lysis of virus-infected target cells can be blocked by applying antibodies to the relevant target cell antigens. Thus far, significant blocking has been achieved with anti-H-2 sera (Burakoff et al. 1976; Koszinowski & Ertl, 1976; Schmitt-Verhulst et al. 1976), but little success has been met with anti-virus sera (Zinkernagel & Doherty 1979). We report here on investigations that define the experimental conditions required for specific blocking with anti-virus sera using ectromelia virus infection as the basic system.

An additional objective was to determine whether anti-virus antibodies contribute to the regulation of primary Tc cell responses to infection in vivo. Though Tc cells seem to be crucial in recovery from ectromelia and other infections, little is known about the regulation of such responses.

METHODS

Animals. All mouse strains (CBA/H, BALB/c, C57BL/6) were bred at the John Curtin School and used at 7 to 10 weeks of age.

Viruses and immunization. Ectromelia and influenza A/JAP viruses were grown, titrated and used for immunization of mice as described elsewhere (Gardner et al. 1974; Yap & Ada, 1977).

Antisera. Convalescent sera were prepared from mice 2 weeks after immunization with either $5 \times 10^4$ p.f.u. attenuated ectromelia virus intravenously (i.v.), $8 \times 10^6$ EID$_{50}$ influenza A/JAP virus i.v., or 250 p.f.u. WE3 strain of lymphocytic choriomeningitis (LCM) virus i.v. Hyperimmune sera were obtained after two further immunizations at 1-week intervals using the same dose of virus. Mice were bled 1 week after the last injection. Rabbit anti-rabbitpox and rabbit anti-vaccinia virus hyperimmune sera were obtained after three inoculations s.c. of $5 \times 10^4$ pock-forming units of either rabbit pox or vaccinia virus at weekly intervals. Rabbits were bled 1 month after the last immunizing infection. All sera were heat-inactivated at 56 °C for 30 min and stored at $-20^\circ$C. The LCM serum was a gift from Dr M. Dunlop and the rabbit sera a gift from Dr K. Lafferty.

In vivo primary responses to ectromelia virus infection have been described before (Gardner et al. 1974).

Memory cultures (in vitro secondary responses). The generation of secondary Tc cells with ectromelia (Gardner & Blanden, 1976) and influenza virus (Yap & Ada, 1977) has been reported in detail. Briefly, spleen cells from mice previously immunized with virus (ectromelia or influenza) were cultured with syngeneic splenic stimulator cells infected with the same virus used for immunization. Two p.f.u. virulent ectromelia virus/cell or 2 50% egg infectious dose (EID$_{50}$) units of influenza virus strain A/JAP per cell were used for the infection of stimulator cells. A stimulator to responder ratio of 1:10 was used for all virus systems. Cultures were set up in tissue culture flasks (Falcon Plastics, 75cm$^2$ growth area) at a final concentration of $2 \times 10^6$ responder cells/ml in a 50 ml/flask. Usually $8 \times 10^7$ responder cells were cultured with $8 \times 10^6$ stimulator cells at 37 °C in an atmosphere of 10% CO$_2$ for 5 days. ( Cultures using ectromelia virus were incubated at 39 °C, a non-permissive temperature for ectromelia replication, which prevents the virus from killing responder cells.) The complete culture medium was Eagle’s minimal essential medium GIBCO Cat. No. F15) with non-essential amino acids, 10% foetal calf serum, $10^{-4}$ M-2-mercaptoethanol and antibiotics.

Cytotoxic assay. The basic method has been fully reported elsewhere (Gardner et al. 1974). In brief, L929, P-815 (Mastocytoma) or 3T3 cells were labelled with $^{91}$Cr for a period of 1 h
in suspension and infected for 1 h in suspension (2 × 10⁶ cells/ml) with either 50 p.f.u./cell of virulent ectromelia virus or 1 EID₅₀/cell of influenza virus. Two × 10⁴ cells/well were distributed in 50 μl samples. After 1 h incubation 50 μl of 1/10 dilution of immune or control sera (if not otherwise specified) were added and incubation was continued for 30 min at 37 °C, before the addition of effector Tc cells in 100 μl samples. Final vol. was 200 μl/well.

The method of preparing macrophage target cells has been described in detail elsewhere (Blanden et al. 1976). The m.o.i. and serum addition was as described above.

Data given have had spontaneous release subtracted and are the means of triplicate assays run at 37 °C for 6 to 16 h. Standard errors of the means were usually less than ±3% and are omitted for clarity. Significance was determined by Student’s t test.

Specific ⁵¹Cr release was calculated according to the formula:

\[
\% \text{ specific } ^{51}\text{Cr} = \frac{\text{test well release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100
\]

% Blocking was calculated as follows:

\[
\% \text{ Blocking} = 100 - \frac{\% \text{ specific } ^{51}\text{Cr release with immune serum}}{\% \text{ specific } ^{51}\text{Cr release with normal serum}} \times 100
\]

Fractionation of serum on sucrose gradients. The method of Baxter (1972) was used for the preparation of linear gradients of 10 to 30% sucrose in isotonic saline. One ml of a 50% dilution of serum in isotonic saline was overlaid on a 12 ml gradient and centrifuged at 30000 rev/min for 20 h at 15 °C in an SW41 rotor. Fractions of 1 ml were collected from the top and dialysed against phosphate-buffered saline (PBS) overnight at 4 °C.

Isolation of IgG. Isolation of IgG on Sepharose A protein columns was performed using the method of Goding (1976) and the Na₂SO₄ precipitation method was used as described by Heide & Schwick (1973).

RESULTS

Kinetics of appearance of serum blocking activity in relation to primary and secondary Tc cell responses to ectromelia virus infection

Sera and spleens of ectromelia virus-infected CBA/H mice were taken from 2 to 21 days p.i. The spleen cells were tested in vitro for primary Tc cell-mediated cytotoxicity against ectromelia virus-infected H-2-compatible L929 target cells and a sample of the spleen cells was cultured in vitro for 5 days at 39 °C with ectromelia virus-infected syngeneic spleen stimulator cells for the detection of secondary Tc cell response capability (memory). The sera of these primed mice was tested for blocking activity in a standard secondary ectromelia-immune Tc cell assay as described in the Methods.

The result of such an experiment is shown in Fig. 1. Primary Tc cell-mediated lysis generated in vivo was highest at day 6 and disappeared by day 10. A primary in vitro response was evident at day 0, i.e. with spleen cells taken from the mice before infection. This capability had disappeared by 2 days p.i., but cells capable of an in vitro response reappeared by day 4 and increased in potential until the end of the experiment. The primary response level (day 0) was exceeded by 14 to 16 days p.i. and presumably is a reflection of memory cell production during the course of the primary response to infection.

Blocking activity in the sera was detectable on days 2 and 4 and reached near plateau levels at day 6 with only slight increases up to day 21. The decline of the primary in vivo Tc cell response after day 6 coincided with the plateau of blocking activity, a finding consistent with, but not proving, a causal relationship.
Primary cytotoxicity (▲—▲), cytotoxicity after 5 days of in vitro stimulation (●—●) and blocking activity in serum (○—○), as tested in a standard secondary ectromelia-immune Tc cell assay, of mice primed for 2 to 21 days with $2 \times 10^4$ p.f.u. ectromelia virus i.v. Primary cytotoxicity was assayed at 30:1 killer to target ratios, and secondary cytotoxicity and blocking at 3:1 killer to target cell ratio on ectromelia-infected L929 cells. Lysis of uninfected controls was negligible.

Table 1. Suppressive effect of hyperimmune serum on primary Tc cell response to ectromelia virus in vivo

<table>
<thead>
<tr>
<th>Day of serum injection* in relation to infection</th>
<th>% specific lysis of ectromelia infected L929 cells</th>
<th>% suppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>No serum</td>
<td>23.9</td>
<td>0</td>
</tr>
<tr>
<td>−3</td>
<td>10.5</td>
<td>56.1</td>
</tr>
<tr>
<td>−1</td>
<td>9.8</td>
<td>59.0</td>
</tr>
<tr>
<td>0</td>
<td>10.2</td>
<td>57.3</td>
</tr>
<tr>
<td>+1</td>
<td>18.1</td>
<td>24.3</td>
</tr>
<tr>
<td>+3</td>
<td>19.8</td>
<td>17.1</td>
</tr>
</tbody>
</table>

* 0.2 ml of a 1/10 dilution of ectromelia hyperimmune serum in Hank's saline was injected i.v.
† Percent $^{51}$Cr release over a 6 h period with spontaneous release subtracted. Killer to target ratio was 15:1. Means of triplicates are given with s.e. of mean never greater than 1.8%. Spontaneous release was 14.6%. Lysis of uninfected L929 was not significantly different from spontaneous release ($P < 0.001$).

Effect of serum on the primary ectromelia virus-immune Tc cell response in vivo

To determine whether virus-immune serum could exert regulatory effects in vivo, the effect of hyperimmune serum on a primary Tc cell response in vivo was investigated by inoculation of mice with serum before and after infection with virus and measuring the primary Tc cell response in the spleen 6 days p.i. Injection of serum from 3 days before till the day of infection significantly reduced the capability of the mouse to mount a primary Tc cell response. Serum administration at a later time had less effect (Table 1). This confirmed earlier results (Pang & Blanden, 1976a) and suggested that virus neutralization or blocking of virus-induced antigens on infected cells in vivo could be a factor in regulating the primary Tc cell response to ectromelia virus infection.
Effect of virus-immune serum on Tc cells

Fig. 2. CBA/H convalescent serum to ectromelia (○, ●) or influenza (□, ■) was tested for blocking activity using secondary ectromelia-immune Tc cells on ectromelia virus-infected L929 targets (○, □) or secondary influenza A/JAP Tc cells on influenza A/JAP virus-infected L929 targets (●, ■). Serum was added to 2 × 10^6 Cr labelled targets at 1/16 to 1/512 dilution 30 min prior to addition of Tc cells. Killer to target cell ratio was 3:1; assay time was 10 h. Specific lysis in the ectromelia and influenza assays without serum was 64.7% and 48.3%, respectively, and lysis of uninfected controls was 1.3% and 0.7%, respectively.

Fig. 3. Sedimentation analysis on a neutral sucrose density gradient of convalescent ectromelia serum. Serum (0.5 ml) diluted with 0.5 ml normal saline was overlaid on a 12 ml 10 to 30% linear sucrose gradient and spun in an SW41 rotor at 30000 rev/min for 20 h at 15 °C. One ml fractions collected from the top of the gradient were dialysed overnight against PBS and tested for blocking activity using secondary ectromelia-immune Tc cells against L929 ectromelia virus-infected targets. Killer to target ratio was 3:1. Specific lysis of the control test without serum was 48.3%. Arrows indicate marker positions of purified mouse IgG and mouse IgM run on separate gradients.

Table 2. Virus specificity of blocking

<table>
<thead>
<tr>
<th>Serum addition*</th>
<th>Ectromelia assay†</th>
<th>Influenza A/JAP assay‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% specific lysis§</td>
<td>% blocking</td>
</tr>
<tr>
<td>None</td>
<td>64.3</td>
<td>0</td>
</tr>
<tr>
<td>Normal CBA/H</td>
<td>60.3</td>
<td>6.1</td>
</tr>
<tr>
<td>CBA/H anti-ectromelia (convalescent)</td>
<td>32.9</td>
<td>48.8</td>
</tr>
<tr>
<td>CBA/H anti-ectromelia (hyperimmune)</td>
<td>28.6</td>
<td>55.5</td>
</tr>
<tr>
<td>CBA/H anti-LCM (convalescent)</td>
<td>66.9</td>
<td>-4.0</td>
</tr>
<tr>
<td>CBA/H anti-influenza A/JAP (hyperimmune)</td>
<td>68.6</td>
<td>-6.7</td>
</tr>
<tr>
<td>CBA/H anti-influenza A/WSN (convalescent)</td>
<td>69.9</td>
<td>-8.9</td>
</tr>
<tr>
<td>Rabbit anti-Rabbitpox (hyperimmune)</td>
<td>42.4</td>
<td>34.0</td>
</tr>
<tr>
<td>Rabbit anti-Vaccinia (hyperimmune)</td>
<td>19.0</td>
<td>70.4</td>
</tr>
</tbody>
</table>

* Serum was added to a final concentration of 1/20 in a total vol. of 0.2 ml.
† Ectromelia virus-infected L929 target cells and CBA/H secondary ectromelia immune Tc cells.
‡ Influenza A/JAP virus-infected L929 target cells and CBA/H secondary influenza A/JAP immune Tc cells.
§ Percent ¹⁴Cr release from virus-infected targets over a 10 h period with spontaneous release subtracted, at a killer to target cell ratio of 3:1.

Virus specificity of blocking

A number of convalescent and hyperimmune sera raised against different viruses were tested for their ability to inhibit lysis by secondary influenza A/JAP and ectromelia-immune Tc cells of influenza A/JAP virus- and ectromelia virus-infected L929 targets respectively.
Table 3. Target cell differences to anti-virus antibody blocking

<table>
<thead>
<tr>
<th>Secondary ectromelia immune T cells</th>
<th>Target*</th>
<th>Normal serum</th>
<th>Ectromelia hyperimmune</th>
<th>% blocking</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBA/H</td>
<td>CBA/H-MΦ§</td>
<td>74:3</td>
<td>72:1</td>
<td>3:0</td>
</tr>
<tr>
<td>L929</td>
<td>CBA/H-3T3</td>
<td>58:3</td>
<td>27:1</td>
<td>53:5</td>
</tr>
<tr>
<td>CBA/H</td>
<td>T cells</td>
<td>48:5</td>
<td>15:7</td>
<td>67:6</td>
</tr>
<tr>
<td>BALB/c</td>
<td>BALB/c-MΦ</td>
<td>82:4</td>
<td>75:4</td>
<td>8:5</td>
</tr>
<tr>
<td>P-815</td>
<td>69:9</td>
<td>60:2</td>
<td>13:9</td>
<td></td>
</tr>
<tr>
<td>BALB/c 3T3</td>
<td>BALB/c 3T3</td>
<td>72:4</td>
<td>31:5</td>
<td>56:5</td>
</tr>
<tr>
<td>BALB/c 3T3 SV40</td>
<td>42:5</td>
<td>16:1</td>
<td>62:1</td>
<td></td>
</tr>
<tr>
<td>C57BL/6</td>
<td>C57BL/6-MΦ</td>
<td>52:8</td>
<td>53:9</td>
<td>—2:1</td>
</tr>
<tr>
<td>C57BL/6 3T3</td>
<td>C57BL/6 3T3</td>
<td>39:6</td>
<td>18:2</td>
<td>54:0</td>
</tr>
</tbody>
</table>

* Targets were used at 2 × 10⁴/well.
† Percent ⁴¹Cr release over an 8 h period with spontaneous release subtracted. Killer to target ratio was 3:1.
‡ Serum was added to give a final concentration of 1/20 in a total vol. of 0.2 ml.
§ Peritoneal macrophages.

(Fig. 2). Virus specificity of blocking was observed throughout when unrelated viruses were used, e.g. LCM and influenza virus-immune sera did not inhibit lysis in the ectromelia system. Sera raised in rabbits against related viruses of the poxvirus group (Downie & MacDonald, 1950) such as rabbitpox virus and vaccinia virus were equally able to inhibit lysis by ectromelia-immune CBA/H Tc cells (Table 2).

Using the ectromelia system we were also able to inhibit lysis of ectromelia virus-infected targets (59.3% inhibition) with hyperimmune serum from mice inoculated with syngeneic, ectromelia virus-infected, glutaraldehyde-fixed spleen cells (data not shown). Again, inhibition of lysis was virus-specific but independent of the mouse strain used for raising the serum.

Characterization of blocking factor in serum

Ectromelia hyperimmune serum was fractionated on a 10 to 30% linear sucrose gradient and dialysed fractions were tested for their ability to inhibit lysis of ectromelia virus-infected L929 targets by secondary ectromelia-immune Tc cells. Marker mouse IgG and IgM were run in separate tubes. The profile of the blocking activity of fractions from the gradient is shown in Fig. 3. Blocking activity coincided with the IgG peak in serum and to a lesser extent with IgM. Furthermore purification of IgG from serum by Na₂SO₄ precipitation, or isolation of IgG on Sepharose A-protein columns, clearly established the blocking factor in hyperimmune serum to be immunoglobulin, mainly IgG.

Target cell type required for efficient blocking

We observed in our initial studies using CBA/H macrophages and L929 cells as targets that only the L929 cell line could be protected from Tc cell-mediated lysis by ectromelia-immune serum. We investigated a number of target cell lines for differences in their susceptibility to blocking. Table 3 summarizes the data obtained using three different strains of mice (CBA/H, BALB/c, C57BL/6) as responders and different H-2 compatible or syngeneic target cell lines. Macrophage targets, irrespective of mouse strain, were not susceptible to blocking, nor were P-815 cells. On the other hand all 3T3 fibroblast lines, L929 cells and an SV40 transformed BALB/c 3T3 line gave good blocking results. In all cases a
CBA/H hyperimmune serum at 1/20 final dilution was used, indicating no mouse strain specificity in the blocking activity of the serum.

DISCUSSION

The ability of serum from mice recovering from virus infection, or of hyperimmune serum, to specifically inhibit Tc cell-mediated lysis of virus-infected target cells in vitro has been demonstrated. This blocking activity was found in the immunoglobulin fractions of hyperimmune serum after purification on Sepharose-A protein columns or by sucrose density gradient centrifugation. The main peak of activity was present in the IgG fraction and minor blocking activity in the IgM fraction, which probably reflects a quantitative rather than qualitative difference. Blocking activity was prominent in the serum of infected mice by the sixth day post-inoculation. This early activity could well be mediated mainly by IgM antibodies, but this remains to be determined.

The ability of immune sera to inhibit Tc cell lysis was virus-specific as far as unrelated virus groups were concerned, but extensive cross-reactivity was observed by virus-immune sera raised to related members of the poxvirus group. Serological cross-reactivity of this group has been well documented (Downie & MacDonald, 1950). Serum raised against infected glutaraldehyde-fixed cells was also able to inhibit lysis. These findings indicate that the same virus-induced antigen(s) on infected cell membranes seen by B cells are physically close to, or a part of, the antigenic pattern seen by Tc cells. The lack of requirement for homology of strain and species between target cells and donors of virus-immune sera suggests that the blocking antibodies recognize virus-specified protein(s). We could find no evidence for blocking attributable to antibodies against host-specified antigens. Thus models for Tc cell recognition involving modified H-2 molecules or derepressed host cell genes seem unlikely.

One interesting aspect of these experiments was the behaviour of different cell types. Fibroblast cell lines (L929 and 3T3) were found to be the only targets which could be protected from Tc cell lysis by virus-immune serum. Macrophage and P-815 mastocytoma target cells were refractory to blocking. The nature of this difference between cells remains to be elucidated but one could envisage greater fluidity of the membrane and/or a faster rate of capping and endocytosis of the antigen-antibody complexes on macrophages and mast cells being a possible factor.

This differential behaviour of target cells used for blocking is important for two reasons. Firstly, the use of L929 cells or fibroblasts in blocking experiments with anti-virus sera is a crucial practical factor that should allow precise identification of the virus-specified proteins involved in Tc cell-recognizable changes in infected cell membranes. Appropriate monoclonal anti-virus antibodies should further improve the efficiency of this approach as they become available. Secondly, infected macrophages and other lymphomyeloid cells are probably the main stimulators of the Tc cell response in vivo, at least with poxviruses (Pang & Blanden, 1976b; Zinkernagel et al. 1978). If such cells are refractory to the blocking effect of anti-virus antibodies on Tc cell recognition, then any regulatory effect of antibodies would have to operate through neutralization of virus infectivity or other mechanisms of virus clearance (Blanden, 1971) rather than blocking.

REFERENCES


A. MULLBACHER AND R. V. BLANDEN


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