Role of early viral surface antigens in cellular immune response to vaccinia virus*

Infection of mice with the vaccinia virus strain WR, Elstree or DIs, a conditional lethal mutant of vaccinia virus, resulted in the generation of vaccinia virus-specific sensitized cytolytic T lymphocytes (CTL). It could be shown by cross-reactivity between the three strains and by inhibition experiments with specific antisera that early vaccinia surface antigens are sufficient for the generation of specific CTL in vivo and for the lysis of infected target cells.

1. Introduction

The specific activity of murine cytolytic T lymphocytes (CTL) sensitized against viruses [1–4], chemically modified cells [5, 6], minor histocompatibility antigens [7] or H–Y antigens [8] is restricted to attacker and target cell homology of the K or D end of the H-2 complex. Killing of modified allogeneic cells is only possible in the tolerant situation of chimeric mice [9]. CTL activities therefore seem to be specific for both H-2 and viral antigens. To obtain further information about possible physiological activities of CTL in the recovery from vaccinia virus infection, the specificity of the sensitizing antigenic structures induced by the virus has to be investigated. Infection with vaccinia virus leads to the destruction of the infected cell. During virus replication and propagation numerous virus-specific antigenic products are synthesized in the cytoplasm and on the surface of the infected cells. Some of these antigens are structural antigens of the virus while others do not seem to be antigenically related to antigens found on the surface of the virus particles [10].

Data reported here were obtained after infection of mice with different strains of vaccinia virus. These strains differed in the expression of viral surface antigens (VSA) on infected cells. Early VSA were sufficient for induction of specific CTL in vivo and for lysis of infected cells in vitro.
2. Materials and methods

2.1. Mice

C3H mice at the age of 6–10 weeks were used throughout, purchased from Bomholtgaard, Ry, Denmark.

2.2. Viruses and immunization

Stocks of vaccinia virus strains WR and Elstree were grown in VERO (Cercopithecus aethiops) kidney cells. Virus titrations were performed on the same cells. Stock solutions of vaccinia virus strain WR and Elstree (Lister strain) contained 1 x 10^6 tissue culture infective dose (TCID)50/ml. Strain DIs, a vaccinia virus mutant provided by Dr. Ueda, Tokyo, was obtained by serial passages of the Dairen-I strain of vaccinia in one-day-old fertile eggs [11]. It is a conditional lethal mutant, producing no clearly recognizable cytopathic effects in Hela, FL or primary monkey kidney cell cultures. The strain is not virulent for newborn, weaning and adult mice and no propagation in mouse tissues or mouse cell lines in vitro can be observed. In cells other than those of chick embryos it fails to induce viral DNA and late protein synthesis, although early antigens detectable by immunofluorescence (IF) and complement fixation are produced [14]. DIs was propagated on 12-day-old fertile eggs at 35–36 °C for 2 days. Titrations were done on primary chicken fibroblasts [12]. DIs was used in a concentration of 1 x 10^5 TCID50/ml. Purification of strain WR was performed according to the method of Joklik [13]. Inactivation of vaccinia virus WR was obtained by incubation at 56 °C for 120 min three times interrupted by a short sonication procedure. Mice were injected intraperitoneally (i.p.) with 1 ml virus suspension six days before harvesting of spleen cells.

2.3. Antisera

Antiserum to strain WR (No. 8): it was obtained from rabbits immunized with WR strain vaccinia. The animals were injected intradermally and after a period of two weeks 3 booster injections were given with an interval of 10 days. Previous to use, the antiserum was absorbed on normal mouse cells. This antiserum contains antibodies with cytotoxic activity for infected cells as well as neutralizing antibodies. With this antiserum VSA can be demonstrated on infected cells by indirect IF. After binding to infected cells, this antibody reacts with cells which are active in antibody-dependent cell-mediated cytolyis (ADCC).

Antiserum to late vaccinia surface antigens, (anti-LVSA): serum No. 8, was extensively absorbed on DIs-infected primary chicken fibroblasts. Absorption end point was determined when no surface staining activity on DIs-infected cells remained. The absorbed serum stained the surface of cells infected with vaccinia strains WR or Elstree by indirect IF. The IF by this antibody may not be directed to a single virus-specific surface antigen, but in this text the antigen(s) which is stained after absorption of DIs antigen activity is termed as LSVA.

Antiserum to early vaccinia surface antigens (anti-EVSA): rabbit serum against EVSA was prepared by injection of crude soluble early antigens of DIs-infected rabbit kidney cells into rabbits. The production of this antibody has been described elsewhere in detail [12]. This antibody has complement fixa-

tion titers against concentrated soluble antigen. It did not neutralize vaccinia virus nor did it stain V antigens of vaccinia-infected cells. As revealed by IF, this serum contained antibodies against EVSA.

Antiserum to structural antigens of the virion (anti-VA): it was obtained from rabbits injected with purified inactivated vaccinia virus. One ODU [15] containing about 64 µg viral protein was injected intramuscularly, a booster injection of the same dose was given 14 days later. This antibody had neutralizing activity but did not bind to the surface of vaccinia virus-infected cells.

All antibodies used had no activities against noninfected control cells. Neutralizing antibody titers were determined by 80 % plaque reduction in VERO cell cultures. Indirect IF was performed as reported previously [15]. Demonstration of DNA synthesis in vaccinia virus-infected cells was performed with 4,6-diamidino-2-phenyl-indol (DAPI) [16]. EVSA and LSVA of cells infected with vaccinia virus were studied by mixed hemagglutination technique [17].

2.4. Cytolytic antibody assay (CA)

The method of Kibler and ter Meulen [18] was modified for L cells as targets and vaccinia strains as infective agents. Guinea pig complement was used in a final concentration of 20 hemolytic units. Test tubes contained 0.05 ml antiserum, 5 x 10^4 51 Cr-labeled erythrocytes in 0.05 ml and 0.1 ml complement. Each assay was run at least in triplicate.

After 4 h incubation at 37 °C in CO2, supernatant and cells were harvested separately and 51Cr release was determined according to the formula:

\[
\% \text{ Lysis} = \frac{51\text{Cr release (Ab + C) - 51\text{Cr release (C alone)}}}{\text{Total } 51\text{Cr incorporated}} \times 100
\]

2.5. Cytolysis inhibition assay (CIA)

Antibodies against cell surface antigens with low cytolytic activity were determined by CIA [19] modified for the determination of VSA. As targets, 1 x 10^4 51 Cr-labeled chicken red blood cells (CRBC), coated with rabbit anti-chicken erythrocyte antibody, were used. Normal mouse spleen cells served as attacker cells in a ratio of A/T of 100:1. To this reaction mixture were added cold inhibitory third-party cells in a ratio of cold cells to erythrocyte targets from 20:1 to 50:1. The inhibitory cells were 1) normal 2) virus-infected L-929 cells, 3) vaccinia-infected L-929 cells coated with anti-vaccinia serum or 4) vaccinia virus-infected L-929 cells + normal mouse control serum. Specific cytolytic inhibition was calculated using the formula:

\[
\% \text{ Specific inhibition} = \frac{\text{inhibition of CRBC lysis in presence of infected inhibitory cells and anti-viral antibody minus inhibition of CRBC lysis in presence of infected inhibitory cells and control serum}}{\text{inhibition of CRBC lysis in presence of infected inhibitory cells and anti-viral antibody}} \times 100
\]

2.6. Cell-mediated cytosis (CMC)

Various amounts of lymphocytes from sensitized mice were incubated with a constant number (1 x 10^4) of 51 Cr-labeled
target cells [4]. The percentage of specific $^{51}$Cr release was determined using the formula:

$$\text{% Specific lysis} = \frac{\text{Specific } ^{51}\text{Cr release by immune cells} - \text{Specific } ^{51}\text{Cr release by normal cells}}{\text{Maximal } ^{51}\text{Cr release}} \times 100$$

The standard deviation (SD) from at least a triplicate assay was calculated. The data are given without SD since, under test conditions used, the SD of percentage lysis was less than 5%.

3. Results

3.1. Virus-specific CMC after sensitization of mice with different vaccinia strains

The virus strains tested varied in the expression of VSA. Characteristics of the three strains used are given in Table 1. Strain WR, the usual test strain, gave positive results in all reactions. Strain DIs did not propagate in L cells, shown by negative DAPI staining and the lack of cytopathic effects, but there was expression of EVSA. Strain Elstree gave mixed hemagglutination results of the single-cell type with anti-EVSA.

Groups of C3H mice were injected i.p. with $1 \times 10^6$ TCID$_{50}$ vaccinia virus strain WR, $1 \times 10^6$ TCID$_{50}$ strain Elstree or $1 \times 10^5$ EID$_{50}$ strain DIs. Six days later spleen lymphocytes were harvested, and the assay was performed with target cells infected with each of the three strains. Results (Table 2) show that mice sensitized to any of the virus strains kill all infected target cells. Also the lymphocytes from mice sensitized to DIs are active in vitro. The mice had been injected with a low dose of DIs virus, and a virus propagation in the mice did not take place and was not necessary for anti-viral sensitization. Injection of DIs-infected L-929 cells led to the production of killer cells, while after injection of allogeneic infected cells there was no anti-viral sensitization.* Furthermore there was a pronounced killing of DIs-infected cells despite the fact that only 60% expressed VSA by means of indirect IF. Lymphocytes from mice sensitized to DIs were also able to lyse targets infected with WR and Elstree strain.

3.2. Inhibition of anti-viral CMC by specific antibodies

The data obtained after infection of mice with strain DIs-infected cells suggest CTL activities against early VSA. Different anti-viral sera were prepared to control these results in inhibition assays (Table 3). These antibodies were tested for inhibitory activities in the anti-viral CMC. Target cells were vaccinia virus WR-infected L-929 cells or noninfected controls; cytolytic effector T cells were harvested from C3H mice sensitized against vaccinia strain WR. Antiserum in 0.05 ml volume was added to the reaction mixture containing 5 x $10^4$ target cells and 5 x $10^6$ effector cells in 0.2 ml

### Table 2. Anti-viral CMC of CTL from mice sensitized with different vaccinia strains

| Sensitization | 
|---|---|---|---|---|
| | % Specific $^{51}$Cr release from L-929 cells infected with $^b$ | WR | DIs | Elstree |
| 1 x $10^6$ TCID$_{50}$ | 45.0 | 41.8 | 15.7 |
| 1 x $10^6$ TCID$_{50}$ Elstree | 18.8 | 27.3 | 15.1 |
| 1 x $10^5$ TCID$_{50}$ DIs | 27.6 | 14.2 | n.t. $^c$ |

* Lymphocyte donors were C3H mice 6 days after sensitization.

### Table 3. Activities of anti-vaccinia sera

<table>
<thead>
<tr>
<th>Serum</th>
<th>Indirect cell surface IF WR</th>
<th>CA</th>
<th>CIA $^b$ (%)</th>
<th>NTC $^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-vaccinia (No. 8)</td>
<td>+</td>
<td>+</td>
<td>1:1024</td>
<td>50–80</td>
</tr>
<tr>
<td>Anti-EVSA</td>
<td>+</td>
<td>+</td>
<td>1:1024</td>
<td>n.t. $^d$</td>
</tr>
<tr>
<td>Anti-LVSA</td>
<td>-</td>
<td>-</td>
<td>1:512</td>
<td>n.t.</td>
</tr>
<tr>
<td>Anti-VA</td>
<td>-</td>
<td>-</td>
<td>1:1024</td>
<td>0–5</td>
</tr>
</tbody>
</table>

* Indirect IF was tested with L-929 cells infected with vaccinia strains WR and DIs 8 h previously.

### Table 1. Characteristics of vaccinia strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>DNA replication in L-929 cells $^a$</th>
<th>Cytopathic effects in L-929 cells</th>
<th>Titer of virus in test $^b$ (TCID$_{50}$)</th>
<th>Indirect hemagglutination with anti-EVSA</th>
<th>Indirect IF on surfaces anti-EVSA anti-LVSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>WR</td>
<td>Yes</td>
<td>Yes</td>
<td>$10^6$</td>
<td>pos.</td>
<td>+++</td>
</tr>
<tr>
<td>Elstree</td>
<td>Yes</td>
<td>Yes</td>
<td>$10^6$ single-cell type</td>
<td>pos.</td>
<td>+++</td>
</tr>
<tr>
<td>DIs</td>
<td>No</td>
<td>No</td>
<td>$10^5$</td>
<td>pos.</td>
<td>++</td>
</tr>
</tbody>
</table>

*a) Tested with DAPI and by indirect IF with anti-VA.
b) WR and Elstree propagated and titrated on VERO cells; DIs propagated on embryonated eggs (chorioalloantois membrane), titrated on primary fibroblasts of 6-day chicken embryos.

---

The data obtained after infection of mice with strain DIs-infected cells suggest CTL activities against early VSA. Different anti-viral sera were prepared to control these results in inhibition assays (Table 3). These antibodies were tested for inhibitory activities in the anti-viral CMC. Target cells were vaccinia virus WR-infected L-929 cells or noninfected controls; cytolytic effector T cells were harvested from C3H mice sensitized against vaccinia strain WR. Antiserum in 0.05 ml volume was added to the reaction mixture containing 5 x $10^4$ target cells and 5 x $10^6$ effector cells in 0.2 ml.
-viral CTL can be inhibited specifically either by H-2 alloantibodies or by anti-viral sera which has been confirmed recently after the addition of alloantibodies often give unreliable results. After capping of SD antigens or of VSA, the lysis by CTL can more effectively be inhibited by addition of alloantibody or anti-viral antibody even during the test period. It cannot be ruled out that the anti-LSVA serum might also have inhibitory activity in higher concentrations. Antibodies raised against inactivated virus particles (anti-VA) have only neutralizing activities while antibodies reactive against EVSA only lyse infected target cells in presence of complement and inhibit anti-viral CTL activity. Therefore virus-neutralizing and CTL inhibitory or lytic properties for infected cells can clearly be separated.

Contact with complete virus particles is not necessary for the generation of CTL since injection of DIs-infected syngeneic cells also leads to effective killer cell production. In parallel, the cytolytic interaction could not be inhibited by large amounts of inactivated vaccinia virus particles. Injection of inactivated virus into mice does not cause CTL production.

For the investigation of the possible biological role of the cytolytic activity of T cells, sensitization and reactivity against EVSA seems to be advantageous. EVSA are expressed as early as 1 h after infection of cells [10] while maturation of complete infective virus needs several hours. Moreover, release of infectious viral particles begins very early and does not start at destruction of the host cell. Vaccinia virus has a tendency to attach to cell surfaces and can spread directly from cell to cell despite specific neutralizing antibody in the surrounding medium [21]. Reacting against EVSA, the CTL prevents viral DNA synthesis and in later stages the spreading of virus. It is tempting to assume that EVSA, whose role in vaccinia replication is unknown, “modify” H-2 antigenic structures and in addition give virus specificity of the H-2-restricted anti-viral CMC. However, experiments performed to demonstrate inhibition of viral proliferation by CTL in vivo have so far been unsuccessful.

We thank Dr. Y. Ueda, Tokyo for supplying DIs virus and anti-ESVA serum and Dr. C. Jungwirth, Würzburg for purification of vaccinia virus WR. The technical assistance of Ms. K.B. Henderson and Ms. S. Siebes is gratefully acknowledged.

Received April 30, 1976; in revised form August 13, 1976.

4. Discussion

Vaccinia virus infection leads to production of several antigens coded by the virus. It has been supposed earlier [10, 12] that the EVSA might play an essential role in cellular immunity. Taking advantage of a conditional lethal mutant strain of vaccinia virus [11] evidence can now be presented that EVSA induced by vaccinia virus give rise to anti-vaccinia CTL. Strain DIs infects mouse cells in vitro and in vivo, but there is no DNA replication shown by virus titration, indirect IF and DNA staining. Injection of DIs-infected cells into mice causes a virus-specific CTL response. Cellular immune response is therefore directed against EVSA expressed on these cells.

Our data give no clear-cut results about the role of LVSA. The strain Elstree is only partially defective in production of EVSA, which can be shown by mixed hemagglutination technique [17]. The CTL activity of mice after sensitization with this strain seems to be lower but does not differ significantly from the response after infection with strain WR or DIs.

The inhibition experiments (Table 4) outline the significance of EVSA in the effector phase of anti-vaccinia CMC. It could be shown in earlier experiments that the activity of the anti-viral CTL can be inhibited specifically either by H-2 alloantibody or by anti-viral sera which has been confirmed recently in the CTL activity against 2,4,6-trinitrophenyl-modified cells [3, 15, 20]. Experiments to show inhibition of CMC after the addition of alloantibodies often give unreliable results. After capping of SD antigens or of VSA, the lysis by
The xenogeneic effect —

Evidence for coparticipation of human monocytes and T lymphocytes in the restoration of nude mouse in vitro response to sheep red blood cells*

The restorative ability of human peripheral blood lymphocyte fractions on nude mouse spleen cell in vitro antibody response to SRBC was studied. Strongly adherent cells (monocytes) enhanced nude cell response, but not to the same extent as the optimal number (10^6) of unfractionated human peripheral blood lymphocytes. T-depleted cells lost their ability to optimally restore the response, while T-enriched cells showed a definite restorative ability. The recombination of adherent cells with T-enriched cells produced an effect comparable to that of unfractionated cells, both in terms of magnitude and dose-response curve. These data suggest that both monocytes and T cells are necessary for an optimal xenogeneic effect in Mishell-Dutton cultures.

1. Introduction

The antibody response is modulated by several kinds of soluble factors. T cells have been shown to produce factors which enhance B cell response to T-dependent antigens, both specifically [1, 2] and nonspecifically [3, 4]. It is probable that both specific and nonspecific factors are simultaneously produced by T cells [5–7] and may act synergistically in the immune response [5]. In addition, activated macrophages produce non-antigen-specific factors capable of augmenting the in vitro antibody response in a T-depleted system [8–10]. The nature and degree to which these factors contribute to the antibody response is not yet fully clear. One of the most important findings in the analysis of the mechanisms of action of such factors has been the demonstration that physiological T–B cell cooperation cannot be demonstrated across a barrier at the major histocompatibility locus [11]. However, nonspecific T cell factors are in most cases [12] active on nonhistocompatible B cells, and an antigen-specific T cell factor has recently been shown to act across an allogeneic barrier [13]. Thus, the biological significance of the histocompatibility-linked restriction to cellular cooperation remains to be fully determined. The study of interactions between xenogeneic cells may provide an additional approach to this problem.

The ability of human peripheral blood lymphocytes (PBL) to produce factors enhancing the in vitro antibody response of mouse cells has been demonstrated in two experimental situations. Rubin et al. [14] have shown that allogeneic mixtures of human lymphoid cell lines, or tetanus toxoid-stimulated normal human PBL could produce such an enhancing factor for normal mouse spleen cell cultures. On the other hand, direct addition of human PBL to cultures of T-depleted mouse spleen cells has been shown to restore their antibody response toward the T-dependent antigen sheep red blood cells (SRBC) [9, 15, 16] using three different sources of T-depleted cells: anti-8+ C-treated cells [9], cells from thymectomized, irradiated and bone marrow-reconstituted mice [15] and cells from nude mice [16]. However, the nature of the human peripheral-blood cell responsible for this effect is controversial. Wood has provided evidence that the restorative capacity of human cells was restricted to monocyctic adherent cells and has shown that short-term monocyte culture supernatants had an enhancing effect [9, 16]. Alternatively, Farrar has demonstrated that adherent cell depletion rather increased the restorative ability of human cells [15] and recently provided evidence that stimulation of human cells with T cell mitogens led to the production of soluble enhancing factors [17].

This work has been designed to investigate the respective role of monocytes and T cells in this xenogeneic effect. The effect of fractionated human peripheral blood cell popula-

---

* This work was supported by grants from INSERM, DGRST and Fondation pour la Recherche Médicale Française.

Correspondence: Pierre Galanaud, Service de Médecine Interne, Hôpital Antoine Béclère, F-92141 Clamart, France