

theoretical and practical implications. The fact that suppression can occur by linked associative recognition argues against an anti-idiotypic form of suppression in this system. Also, it should be possible to suppress DTH to an antigen A by immunizing with an appropriate antigen B and then reimmunizing with the AB conjugate. Preliminary results indicate that an established DTH to HRBC can be suppressed by first immunizing with HCY and then with HCY coupled to HRBC. This may have practical applications in situations where it would be advantageous to suppress a DTH response.

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U. Koszinowski and Hildegund Ertl

Institute of Hygiene, University of
Göttingen, Göttingen

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 *in vitro*.

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

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Correspondence: Ulrich Koszinowski, Department of Zoology, University College London, Gower Street, London WC1E 6BT, GB

Abbreviations: ADCC: Antibody-dependent cell-mediated cytotoxicity
CA: Cytolytic antibody assay CIA: Cytotoxicity inhibition assay CMC:
Cell-mediated cytotoxicity CTL: Cytolytic T lymphocytes DAPI: 4,6-
Diamidino-2-phenylindole EVSA: Early viral surface antigens IF:
Immunofluorescence LCM: Lymphocytic choriomeningitis LVSA:
Late viral surface antigens TCID: Tissue culture infective dose VA:
Antigens of the virus particle VSA: Viral surface antigens DI:
Dairen-I mutant strain of vaccinia virus CRBC: Chicken red blood cells

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 particle [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 1×10^6 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 virus 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 1×10^5 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 cytotoxicity (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 LSA.

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 LVSA 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, 5×10^4 ⁵¹Cr-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:

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

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, 1×10^4 ⁵¹Cr-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:

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

2.6. Cell-mediated cytotoxicity (CMC)

Various amounts of lymphocytes from sensitized mice were incubated with a constant number (1×10^4) of ⁵¹Cr-labeled

target cells [4]. The percentage of specific ⁵¹Cr release was determined using the formula:

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

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 x 10⁶ TCID₅₀ vaccinia virus strain WR, 1 x 10⁶ TCID₅₀ strain Elstree or 1 x 10⁵ EID₅₀ 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 also 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.

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Table 1. Characteristics of vaccinia strains

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

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 (chorioallantois membrane), titrated on primary fibroblasts of 6-day chicken embryos.

Table 2. Anti-viral CMC of CTL from mice sensitized with different vaccinia strains^{a)}

Sensitization		% Specific ⁵¹ Cr release from L-929 cells infected with ^{b)}		Elstree
		DIs	WR	
1 x 10 ⁶ TCID ₅₀	WR	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)}

- a) Lymphocyte donors were C3H mice 6 days after sensitization.
- b) Background lysis ranged between 20-25 %; assay time 14 h, ratio A/T 100:1.
- c) n.t. = not tested.

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 10⁴ target cells and 5 x 10⁶ effector cells in 0.2 ml

Table 3. Activities of anti-vaccinia sera

Serum	Indirect cell surface IF WR	IF DIs ^{a)}	CA	CIA ^{b)} (%)	NT ^{c)}
Anti-vaccinia (No. 8)	+	+	1:1024	50-80	1:32
Anti-EVSA	+	+	1:1024	n.t. ^{d)}	n.t.
Anti-LVSA	+	-	1:512	n.t.	n.t.
Anti-VA	-	-	1:>4	0-5	1:256

- a) Indirect IF was tested with L-929 cells infected with vaccinia strains WR and DIs 8 h previously.
- b) Inhibitory activity of cold vaccinia WR-infected L-929 cells coated with anti-vaccinia serum No. 8 on antibody-dependent cell-mediated lysis of antibody-coated ⁵¹Cr-labeled CRBC could be demonstrated up to a dilution of 1:1024 of serum No. 8.
- c) Neutralizing activity was tested on VERO cell monolayers.
- d) n.t. = not tested.

volume. The results are shown in Table 4. We found significant inhibition of CMC using serum No. 8 and anti-ESVA serum. Anti-LSVA serum and serum directed against structural antigens (anti-VA) had no significant inhibitory activities. The inhibition test with 1 optical density unit of inactivated vaccinia antigen gave negative results, after addition of about 1.2×10^{10} vaccinia virus particles there was no inhibition of anti-viral CMC. The virus-specific receptor site of the cytolytic T cell does not seem to recognize antigens of the vaccinia virus particle. The data confirm the finding that CTL recognize early vaccinia surface antigens.

Table 4. Anti-viral CMC in presence of specific antibodies or inactivated virus^{a)}

Serum	Specific lysis (%)
None	27.4
Normal serum	24.8
Anti-vaccinia (No. 8)	15.0 ^{b)}
Anti-EVSA	17.1 ^{b)}
Anti-LVSA	23.7
Anti-VA	25.8
2×10^{10} vaccinia particles	32.4

a) Donors of vaccinia-immune spleen cells were C3H mice infected 6 days previously with vaccinia WR. Target cells were WR-infected L-929 cells. Ratio A/T 100:1. Incubation time 12 h. Background lysis less than 25 %.

b) Significant lower specific ^{51}Cr release ($P < 0.05$).

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 alloantisera 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

CTL can more effectively be inhibited by addition of allo-antibody 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 infective viral particles begins very early and does not lead at once to 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 vitro* have so far been unsuccessful.

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T. Estroff^o, P. Galanaud, J. Dormont,
Christine Wallon and G. Tchernia

Groupe de Recherche INSERM U 131,
Service de Médecine Interne, Hôpital
Antoine Bécclère, Clamart

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 recent-

ly 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- Θ + 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 monocyte 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-

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

Abbreviations: SRBC: Sheep red blood cells MEM: Minimum essential medium AFC: Antibody-forming cells PBL: Peripheral blood lymphomonocytic cells UHPBL: Unfractionated human peripheral blood lymphomonocytic cells T-enriched/depleted: Thymus-dependent cells enriched/depleted E rosette: SRBC rosette FBS: Fetal bovine serum HBSS: Hanks' balanced salt solution