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Virus-Specific T-Cell Sensitization

Requirements for Vaccinia Virus-Specific T-Cell Sensitization in vivo*

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Abstract. Syngeneic, semiallogeneic, or allogeneic spleen lymphocytes were transferred into *nu/nu* BALB/c mice, which were infected with vaccinia virus. Specific sensitization of transferred thymus-derived cells was determined in vivo by mean survival time and virus titer in the spleen six days after infection, and in vitro by cell-mediated cytolysis of vaccinia virus-infected syngeneic target cells. Virus-specific sensitization took place only after transfer of syngeneic or semiallogeneic spleen lymphocytes; allogeneic lymphocytes had no influence on mean survival time or virus titer and showed no virus-specific cytolytic activity in vitro. Infection of mice with vaccinia virus-strain WR, Elstree, DIs, or DIs-infected syngeneic fibroblasts resulted in the generation of virus-specific effector cells, while injection of a high amount of inactivated virus particles caused no sensitization. These results suggest H-2 homology for production of virus-specific effector cells. Propagation of virus is not necessary, since early surface antigens, combined with syngeneic H-2 antigens, suffice for sensitization of cytolytic T lymphocytes.

Introduction

The infection of mice with certain viruses such as the LCM virus (Zinkernagel and Doherty 1974), poxviruses (Gardner *et al.* 1975, Koszinowski and Thomssen 1975), or Sendai virus (Ertl and Koszinowski 1976) results in the generation of cytolytic T lymphocytes, which are able to kill in vitro cells infected with the virus used for sensitization. The activity of CTLs sensitized against viruses, chemically modified cells (Shearer *et al.* 1975), minor histocompatibility antigens (Bevan 1975, Gordon *et al.* 1975) is restricted to attacker- and target-cell homol-

* Abbreviations used in this paper are as follows: CMC, cell-mediated cytolysis; CTL, cytolytic T lymphocyte; LCM, lymphocytic choriomeningitis; MHC, major histocompatibility complex; MST, mean survival time; T cell, thymus-derived cell; $TCID_{50}$, 50 percent tissue culture infective dose.

ogy of the *K* or *D* end of the major histocompatibility complex. Two hypotheses have been postulated to explain this H-2 restriction (Doherty and Zinkernagel 1975). The physiological interaction theory needs a T cell-specific antigen, such as a viral surface antigen, and an interaction between identical H-2 structures on effector and target cell for recognition and lysis. The altered self theory proposes an alteration of H-2 antigenic determinants by virus infection or by chemical treatment of cells. Recent findings in chimeric mice (Zinkernagel 1976) favor the latter theory. Another hypothesis, which proposes a "dual recognition" by two T-cell receptors specific for a (modified) *K*- or *D*-region product and a viral surface antigen as a requirement for target cell lysis, should be considered. Experiments, which show an inhibition of CMC by antiviral antibodies (Koszinowski and Thomssen 1975) are in agreement with this concept.

The role of H-2 antigenic structures in the sensitization phase is still unknown. Data reported here were obtained after infection of *nu/nu* BALB/c mice which were inoculated with syngeneic, semiallogeneic, or allogeneic spleen cells. A specific sensitization, measured in vivo by the mean survival time and virus titer in the spleens and in vitro by cytolytic activity of CTL, was dependent on the homology of the *K* and *D* ends of the *H-2* complex between lymphocytes and infected cells.

To obtain further information about the viral antigens for sensitization of T cells, mice were infected with different virus strains or with inactivated vaccinia virus particles. The three strains we used differed in the expression of viral surface antigens on infected cells. Early vaccinia surface antigens were sufficient for induction of specific CTL in vivo.

Materials and Methods

Animals. Mice of the inbred strains C3H ($H\text{-}2^k$), DBA/2 ($H\text{-}2^d$), A/J ($H\text{-}2^a$), and *nu/nu* Balb/c ($H\text{-}2^d$) at the age of 6–10 weeks were used throughout. They were purchased from Bomholtgard, Ry, Denmark.

Viruses. Vaccinia virus, strain WR and strain Elstree (Lister strain), was propagated in Vero cells (monkey kidney cells) and used in a concentration of $10^{5.5}$ $TCID_{50}$/ml. Strain DIs, a conditional lethal mutant of the DIE strain (Tagaya *et al.* 1961), kindly provided by Dr. Y. Ueda, Tokyo, Japan, was propagated in 12-day-old fertile chicken eggs at 37°C for 2 days. DIs virus was used in a concentration of 1×10^5 $TCID_{50}$/ml.

Virus Purification. Virus was purified by the method of Joklik (1962).

Virus Titration. WR and Elstree viruses were titrated on Vero cells. Titration for DIs was done on primary chicken fibroblasts (Tagaya *et al.* 1974). $TCID_{50}$ was determined according to the method of Reed and Muench (1938).

Virus Inactivation. Vaccinia virus infectivity was inactivated without loss of antigenicity by incubation at 56°C for 120 minutes, interrupted 3 times by a short sonication procedure.

Target Cells. L-929 fibroblasts ($H\text{-}2^k$), obtained from Dr. Lehmann-Grube, Hamburg, and mastocytoma P-815–X-2 cells were cultivated in Eagle's minimal essential medium, supplemented with 10% inactivated calf serum and 100 μg/ml streptomycin and penicillin.

Infection and Radioactive Labeling of Target Cells. About 5×10^5 L-929 cells growing as monolayer cultures in glass bottles were incubated with 10 ml vaccinia virus suspension on a rocker platform for 2 hours. The virus suspension was then replaced by fresh serum-free medium, and about 16–18 hours later, cells were trypsinized and incubated with 100 μCi ^{51}Cr (sodium chromate, Amersham, Buchler, Braunschweig, 100 to 200 mCi/mg Cr No CJS1P) in a volume of 1 ml for 1 hour. After washing 3 times to remove unbound radioactivity, the cells were used in cytolytic assays. P-815 cells were infected with about 5 $TCID_{50}$ per cell for 2 hours, washed twice, and labeled with ^{51}Cr for 1 hour.

Effector Cells. Mice were infected intraperitoneally with $10^{5.5}$ $TCID_{50}$ (strains WR and Elstree), 10^5 $TCID_{50}$ (strain DIs), inactivated vaccinia virus (10^{10} $TCID_{50}$ before inactivation), or 1×10^6 L-929 cells infected with DIs virus. Nude BALB/c mice were inoculated intravenously with 5×10^7 Ficoll-purified spleen cells of C3H, DBA/2, or A/J mice. Six days later, spleens were removed and lymphocytes were prepared.

Cell-Mediated Cytolysis (CMC). The activity of spleen lymphocytes was tested in an 18-hour Cr-release assay as described earlier (Koszinowski and Thomssen 1975). Briefly, 5×10^6 spleen cells from immunized or normal mice were incubated in microplates with 5×10^4 normal or vaccinia virus-infected ^{51}Cr-labeled target cells in a volume of 0.2 ml at 37°C in a humidified atmosphere with 7% CO_2. Eighteen hours later, supernatant and cell pellet were separated and the percentage of Cr release was determined. Percentage of specific lysis was calculated using the following formula:

Percent specific lysis = Percent Cr release in presence of sensitized lymphocytes
− Percent Cr release in presence of normal lymphocytes

Results

Mean Survival Time of Nude Mice Infected with Vaccinia Virus and Inoculated with Syngeneic, Semiallogeneic, or Allogeneic Spleen Lymphocytes

Normal inbred mice survive a vaccinia virus infection without obvious symptoms. Nude mice, which are characterized by a congenital lack of thymus-derived cells (T cells) died about two weeks after the infection. Inoculation of nude mice with 5×10^7 allogeneic spleen lymphocytes did not prolong the survival time. Only syngeneic (or semiallogeneic) spleen cells extended the MST significantly (Fig. 1).

Vaccinia Virus Titer in the Spleens of Nude Mice Six Days After Vaccinia Virus Infection

Groups of three *nu/nu* BALB/c mice were infected with $1 \times 10^{5.5}$ $TCID_{50}$ vaccinia virus (strain WR) and inoculated with 5×10^7 syngeneic (DBA/2), semiallogeneic (A), or allogeneic (C3H) spleen lymphocytes. Six days later, spleens were removed and the virus content was estimated by titration on Vero cell monolayers. As is shown in Table 1, spleens of nude mice infected with vaccinia virus contained about $10^{3.2}$ $TCID_{50}$ virus. Spleens of infected animals, which were inoculated with allogeneic spleen lymphocytes, contained comparable amounts of virus, while in spleens of mice which received syngeneic or semiallo-

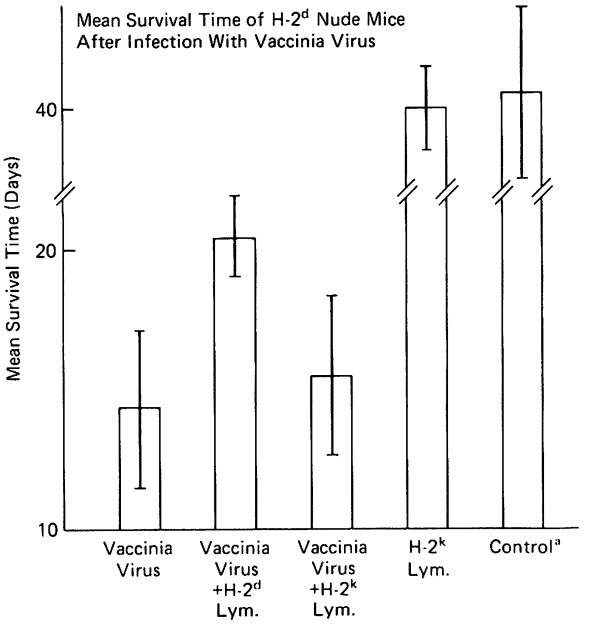

Fig. 1. Groups of *15nu/nu* BALB/c mice were infected with $1 \times 10^{5.5}$ $TCID_{50}$ vaccinia virus and injected simultanously with 5×10^7 allogeneic (C3H), or syngeneic spleen lymphocytes. Controls received medium or allogeneic spleen cells alone. [a] Lethality of untreated mice

Table 1. Reduction of Vaccinia Virus Titer in the Spleens of Nude Mice[a]

Origin of Spleens	Virus Titer
Nude mice (*H-2d*) + $10^{5.5}$ $TCID_{50}$ Vaccinia virus	$10^{3.2}$ $TCID_{50}$
Nude mice (*H-2d*) + $10^{5.5}$ $TCID_{50}$ Vaccinia virus + A (*H-2a*) spleen cells	0
Nude mice (*H-2d*) + $10^{5.5}$ $TCID_{50}$ Vaccinia virus + C3H (*H-2k*) spleen cells	$10^{2.6}$ $TCID_{50}$
Nude mice (*H-2d*) + $10^{5.5}$ $TCID_{50}$ Vaccinia virus + DBA/2 (*H-2d*) spleen cells	0

[a] The vaccinia virus content of spleen cells of three *nu/nu* BALB/c mice infected six days previously with $1 \times 10^{5.5}$ $TCID_{50}$ vaccinia virus and injected with 1×10^7 syngeneic, semiallogeneic, or allogeneic spleen lymphocytes was estimated by titration on Vero cells. $TCID_{50}$ was determined by the method of Reed and Muench (1938).

geneic spleen lymphocytes, no infectious virus was demonstrable six days after infection.

Cell-Mediated Cytolysis by Lymphocytes of nu/nu BALB/c Mice Infected with Vaccinia Virus and Substituted with Syngeneic, Semiallogeneic, or Allogeneic Spleen Lymphocytes

Nude mice were not able to produce cytolytic lymphocytes after infection with vaccinia virus (Table 2). After transfer of 5×10^7 allogeneic spleen lymphocytes, no virus-specific killer cells against virus-infected target cells were generated;

Table 2. Cell-Mediated Cytolysis of Normal and Vaccinia Virus-Infected P-815 Target Cells[a]

Origin of Lymphocytes	Percent Specific Lysis	
	P-815	P-815-Infected
nu/nu (*H-2*^{d}) → vaccinia virus	1.3 ± 2.3	4.4 ± 4.3
nu/nu (*H-2*^{d}) + A (*H-2*^{a}) spleen cells	2.1 ± 1.4	4.3 ± 2.3
nu/nu (*H-2*^{d}) + A (*H-2*^{a}) spleen cells → vaccinia virus	2.9 ± 1.0	17.7 ± 6.1
nu/nu (*H-2*^{d}) + C3H (*H-2*^{k}) spleen cells	11.1 ± 4.5	0.1 ± 4.1
nu/nu (*H-2*^{d}) + C3H (*H-2*^{k}) spleen cells → vaccinia virus	9.8 ± 2.4	0.5 ± 4.0
nu/nu (*H-2*^{d}) + DBA/2 (*H-2*^{d}) spleen cells	2.6 ± 2.5	0.7 ± 2.2
nu/nu (*H-2*^{d}) + DBA/2 (*H-2*^{d}) spleen cells → vaccinia virus	1.3 ± 0.6	22.8 ± 3.9

[a] The cytolytic activity of spleen lymphocytes of *nu/nu* BALB/c mice infected six days previously with vaccinia virus and injected with 1×10^7 syngeneic, semiallogeneic, or allogeneic spleen cells was tested on normal and infected P-815 target cells. Assay time: 18 hours. Attacker cell to target cell ratio: 100:1.

only a weak allograft reaction against noninfected P-815 cells could be observed. Syngeneic or semiallogeneic-transferred spleen lymphocytes were able to kill only vaccinia virus-infected target cells and had no cytolytic effect on noninfected targets. Using normal and vaccinia virus-infected L-929 cells as controls, no specific lysis by any group of CTL was observed.

Role of Different Vaccinia Virus Antigens in Sensitization of Antiviral CTL

Groups of three C3H mice were infected by intraperitoneal injection of $1 \times 10^{5.5}$ $TCID_{50}$ vaccinia virus, strain WR or Elstree; $1 \times 10^{5.0}$ $TCID_{50}$ DIs virus; heat-inactivated purified virus (1×10^{10} $TCID_{50}$ before inactivation); or 1×10^7 DIs-infected L-929 fibroblasts.

The three vaccinia virus strains vary in the expression of viral surface antigens. Strain WR, our standard test strain, propagates in mouse cells and expresses early and late surface antigens on the infected cells. Strain Elstree is

Table 3. CMC by Lymphocytes from Mice Sensitized with Different Vaccinia Virus Strains and Viral Antigens[a]

Origin of Lymphocytes	Percent Specific Lysis of WR-Infected L-929 cells
C3H → $1 \times 10^{5.5}$ $TCID_{50}$ WR	45.0 ± 2.6
C3H → $1 \times 10^{5.5}$ $TCID_{50}$ Elstree	18.8 ± 1.2
C3H → $1 \times 10^{5.0}$ $TCID_{50}$ DIs	27.6 ± 2.4
C3H → inactivated vaccinia virus	1.4 ± 1.9
C3H → L-929, DIs-infected	16.9 ± 1.3

[a] The cytolytic activity of spleen lymphocytes of C3H mice infected six days previously with $1 \times 10^{5.5}$ $TCID_{50}$ vaccinia virus, strain WR or Elstree; $1 \times 10^{5.0}$ $TCID_{50}$, DIs virus; 1×10^7 DIs-infected L-929 cells; or 1×10^{10} $TCID_{50}$ heat-inactivated vaccinia virus (WR) was tested on vaccinia virus (WR)-infected L-929 target cells. Assay time: 18 hours. Attacker cell to target cell ratio: 100:1.

defective in the production of early surface antigens. Strain DIs does not propagate in mouse cells but expresses early surface antigens.

Lymphocytes of mice infected with any of these virus strains were able to kill vaccinia virus-infected syngeneic target cells (L-929 infected with WR virus, Table 3). Also, the injection of DIs virus-infected syngeneic fibroblasts led to the production of CTL, while the injection of a high dose of inactivated purified virus particles caused no antiviral sensitization of T lymphocytes.

Discussion

T cell-mediated immunity is essential for recovery from poxvirus infection (Blanden 1974, Blanden *et al.* 1975). Macrophages, B cells, or antibodies do not seem to suffice. This was shown in nude mice, which are characterized by a congenital lack of thymus-derived lymphocytes. In contrast to normal mice, these animals died about one to two weeks after a vaccinia virus infection.

One important role of sensitized T lymphocytes may be the control of virus multiplication. T cells are probably not able to reduce the number of virus particles (Blanden 1971). There is some evidence for virus clearance by macrophages, which are activated by sensitized immune lymphocytes (Koszinowski *et al.* 1975). Infected cells, which express early viral surface antigens, are lysable by CTL (Koszinowski and Ertl 1976). Early viral surface antigens are synthetized about one hour after the infection, while the release of complete infective virus particles requires 16 to 20 hours. CTL prevent viral DNA synthesis and virus multiplication by attacking cells bearing early viral surface antigens.

For sensitization of T lymphocytes, early viral surface antigens were sufficient, as has been proposed by other authors (Ueda and Tagaya 1973). Infection of mice with a high dose of inactivated virus was ineffective, so the stimulus for in vivo induction of cytolytic T lymphocyte production seems to be the virus-infected cell, rather than the free virion. Structural antigens may be essential for activation of B cells, which produce neutralizing antibodies. The role of late surface antigens, which are expressed on the cell surface during viral DNA synthesis, remains unexplained under the experimental conditions, since the Elstree strain is only partially defective in production of early surface antigens (Ito and Barron 1972).

The cytolytic activity of T cells is characterized by two restrictions. First, their action is virus-specific; no crossreactivity occurs between such different viruses as LCM virus, vaccinia virus, or Sendai virus (Ertl and Koszinowski 1976). Second, for efficient lysis, donors of the cytolytic T lymphocytes and infected cells must be identical at the *K* or *D* region of the *H-2* gene complex, since CTL discriminate even point mutations within the *K* region (Doherty *et al.* 1976). H-2 homology is also necessary for the production of virus-specific effector cells, as was shown by transfer of syngeneic, semiallogeneic, or allogeneic spleen cells into vaccinia virus-infected nude mice.

These data agree with the modified self hypothesis. One may speculate that early viral surface antigens modify H-2 antigenic structures. Recirculating T lymphocytes control the integrity of H-2 antigens; cells bearing altered or un-

known (allogeneic or xenogeneic) H-2 antigens lead to clonal expansion of T lymphocytes, which eliminate these cells.

Tansfer of syngeneic or semiallogeneic lymphocytes into infected nude mice caused increased MST and virus clearance in vivo. In vitro, the transferred lymphocytes lysed virus-infected syngeneic target cells. Induction of virus-specific CTLs across the H-2 barrier was possible in chimeric mice (Zinkernagel 1976). In contrast to lymphocytes, which were transferred into allogeneic nude mice, CTLs from chimeric mice are tolerant to the allogeneic target cells, which may partially explain the differing results. The in vitro activity of intolerant allogeneic sensitized CTLs was rather weak. Neither virus-infected allogeneic nor normal or infected syngeneic target cells were lysed. Transfer of allogeneic spleen cells had no influence on the progress of virus infection in vivo: MST was not prolonged and virus titer in these mice was not reduced significantly. One reason for the failure of protection by transferred allogeneic T cells may be the lack of generation of cytolytic T cells. Furthermore, it may indicate that collaboration between T cells and other immune mechanisms, which cause clearance of infective virus under syngeneic or semiallogeneic conditions, is impossible in a complete allogeneic situation.

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