IMMUNE RESPONSE IN HUMANS AFTER VACCINATION WITH VACCINIA VIRUS: GENERATION OF A VIRUS-SPECIFIC CYTOTOXIC ACTIVITY BY HUMAN PERIPHERAL LYMPHOCYTES*

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The outcome of a virus infection mainly relates to the respective characteristics of the viruses, the cells they replicate in, and the immune system of the host (1–4). The immune response of man is usually analyzed in terms of humoral or cellular reactions. Much data have accumulated on humoral responses to viral infections; in fact, a rise in antibody titer against a given virus or its antigenic components is currently used to identify the agents causing a patient's infection (5). Specific anti-viral antibodies are also a major factor in inhibiting the spread of viral infection, resulting in the patient's recovery from a primary infection and the prevention of reinfection with the same agent (6–8). In vitro, specific antibodies can reduce the infectivity of most viruses that bud from cells' plasma membranes and, when fresh serum is added to the culture as a source of complement, can lyse virus-infected cells (9, 10). In cooperation with lymphoid cells isolated from human peripheral blood, anti-viral antibodies lyse virus-infected target cells in vitro, although neither reactant alone is lytically efficient (11–14).

Yet, three lines of evidence strongly suggest that cell-mediated immunity is involved in recovery from viral infection in man. First, delayed-type hypersensitivity (DTH)[1] reactions can be generated after many virus infections. Jenner probably observed such a reaction in a patient previously exposed to cowpox and variolated (15). Others have reported DTH reactions in humans infected with a variety of viruses (16, 17). Second, patients with congenital or acquired gamma globulin deficiencies recover normally from infections with measles and varicella viruses. In contrast, children having profound T-cell defects associated with thymic lymphopenia or Wiscott-Aldrich syndrome develop

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[1] *Abbreviations used in this paper:* ADCC, antibody-dependent cell cytotoxicity; CMV, cytomegalovirus; DTH, delayed-type hypersensitivity; EA, ox erythrocytes sensitized with rabbit IgG anti-ox erythrocyte; FCS, fetal calf serum; MOI, multiplicity of infection, NP-40, Nonidet P-40; ORBC, ox erythrocyte; PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline; PFU, plaque-forming units; SRBC, sheep red blood cells.

major complications like giant cell pneumonia and encephalitis after measles virus infection (18–20). Vaccination of these children with vaccinia virus has led to generalized vaccinia infection (21). Third, experiments involving either virus-infected murine target cells mixed with thymus-derived (T) lymphocytes from infected mice or intact mice undergoing experimental depletion and reconstitution of T cells both provide clear evidence of a cellular immune defense mechanism against a variety of virus infections like ectromelia, vaccinia, and lymphocytic choriomeningitis (22–24). Murine cytotoxic T cells can also be generated in vitro during a secondary immune response to virus (25).

Observations made in mice suggest directions for seeking T-lymphocyte-mediated responses in humans. The time-course of murine T-cell responses is sharply limited after primary infection, since the number of cytotoxic T cells is maximal 5–7 days after infection and negligible by the 12th day. Moreover, cytotoxic T cells lyse virus-infected murine target cells only when both cell types share at least one gene product of the *K* or *D* region of the major murine histocompatibility (*H-2*) complex (26, 27).

With this background information, we decided to investigate cell-mediated cytotoxic responses in humans against virus-infected target cells and determine whether this reaction is related to HLA specificities, the human counterpart of murine *K* and *D* specificities. We wanted to see whether a similar time-course to raise cytotoxic cells and a genetic restriction occurred in man. We chose to immunize volunteers with vaccinia virus because its effects are relatively inocuous and recovery is uncomplicated. Further, vaccinia virus, and the closely related ectromelia virus, are known to elicit well-defined cytotoxic T-cell responses with *H-2* restriction in mice (22, 27).

Materials and Methods

Immunization of Human Subjects. Nonimmune (normal) peripheral blood lymphoid cells were obtained from blood of human volunteers before immunization and from other volunteers who were not immunized. Immune cells came from 12 normal adults given vaccinia virus vaccine. These included nine males and three females, 21–43 yr of age, all free of skin disease, allergic history, and ocular infection. All subjects had been vaccinated with vaccinia virus during childhood, and two had been revaccinated 2 or more years before this study. For this study, each subject underwent a 2 cm^2 scarification with smallpox vaccine (Lederle Laboratories, Pearl River, N. Y.) in the area of the left deltoid muscle, and 36–48 h later induration and redness developed with resolution within 7–10 days. Vesicles and regional adenopathy appeared by 72 h. No adverse reactions due to vaccination occurred. For most subjects, 30–50 ml of blood was obtained from the antecubital vein on the day of vaccination (day 0) and on days 1, 3, 5, 7, and 12 after vaccination; others gave blood on days 0, 6, 7, 8, and 21. Drawn blood was transferred sterily to 50-ml plastic tubes containing heparin (20 U heparin/ml blood).

Peripheral Blood Lymphocytes (PBL). Peripheral blood lymphomonocytes were obtained after Ficoll-Hypaque gradients (28). Monocytes were removed by adherence to plastic dishes after 1 h at 37°C in 5% CO_2. As tested by eosin uptake, more than 95% of monocytes were removed by this procedure. PBL were separated into T cells and T-depleted PBL by using the ability of T cells to make rosettes with sheep erythrocytes (SRBC) treated with neuraminidase (13, 29). In most experiments the T cells in the pellet obtained from the Ficoll-Hypaque gradient were carefully resuspended with 5–10 ml of medium [RPMI 1640 + 20% fetal calf serum (FCS)], and the T-cell rosettes were rerun through a second Ficoll-Hypaque gradient. T cells were freed from SRBC by warming resuspended cells at 37°C for 20 min, shaking vigorously, and reapplying to a Ficoll-Hypaque gradient or by lysis of the SRBC after briefly resuspending the pellet in a low ionic acid solution [1/3 Eagle's minimal essential medium (MEM) and 2/3 distilled water] followed by addition of MEM containing 10% FCS and sodium bicarbonate. In both cases T cells were washed twice after manipulation. The T-cell population recovered was greater than 98% pure on the basis of rerosetting with SRBC treated with neuraminidase and by the absence of surface IgG markers

as tested by immunofluorescence (less than one cell positive per 200) using polyvalent fluoresceinated goat anti-human IgG.

In some experiments the PBL were segregated into populations of Fc receptor-positive and negative cells by using ox erythrocytes (ORBC) treated with a subagglutinating dose of rabbit IgG antisera to ORBC (EA) (30). In brief, 5×10^6 PBL in RPMI 1640 supplemented with 10% FCS were incubated at a ratio of 1:25 with EA for 5 min at room temperature and spun at 1,000 rpm for 10 min in an International PR2 centrifuge. After an incubation of 30 min at room temperature and gentle resuspension, the mixture was layered on the top of a Ficoll-Hypaque gradient and spun for 15 min at 2,200 rpm using an International PR-2 centrifuge (Damon Int. Equip. Corp., Needham Heights, Mass.). Fc-negative PBL at the interface were collected, washed twice, counted, and resuspended at the desired concentration. Fc-positive cells were found in the pellet, resuspended in the same medium, and rerun through a Ficoll-Hypaque gradient, after which the ORBC were lysed by treatment with the low ionic acid solution. The lymphoid cells were pelleted and resuspended in MEM supplemented with 10% FCS and bicarbonate. After 60 min incubation at room temperature, lymphoid cells were washed twice and resuspended at the desired concentration. The subpopulations of Fc-positive and negative cells were each more than 95% pure as judged by rerosetting with EA. In various experiments the percent of Fc-positive cells in PBL varied from 21 to 40%. The T-cell and T-depleted populations described above were also segregated into Fc-positive and negative cells with the above method.

Virus. The WR strain of vaccinia virus originally passed in L 929 cells was a gift from Dr. W. K. Joklik, Duke University, Durham, N. C. This virus was used to infect HeLa cells grown in suspension in 2,000-ml flasks containing 400 ml of Spinner's medium supplemented by 10% FCS, 2% glutamine, and antibiotics (penicillin, 75 U/ml; streptomycin, 75 μg/ml). The cells were infected at a multiplicity of infection (MOI) of 0.5–1. Supernatant fluid was removed after 48 h, and vaccinia virus was isolated from the cells, sonicated, and banded in a sucrose density gradient (31). The virus pool used titered 2.5×10^9 plaque-forming units (PFU)/ml on Vero cells (32). Wild-type Edmonston measles virus was obtained from the American Type Culture Collection, Rockville, Md. and passed on HeLa cells (33).

Cell Lines. Skin fibroblasts were obtained from biopsies taken at the forearms of eight of the vaccinated volunteers, and processed as previously described (13). HeLa cells were obtained from Eric Stanbridge, Department of Microbiology, University of California Medical School, Irvine, Calif. and were devoid of mycoplasma contamination. Another line of HeLa cells persistently infected with measles virus was raised as previously described (33). The Vero cells (monkey kidney) and the mouse cell line L 929 were initially obtained from Flow Laboratories, Inc., Rockville, Md. and propagated in our laboratory. All four cell lines were grown as monolayers in 75 cm^2 Falcon plastic flasks at 37°C in a moist atmosphere containing 5% CO_2. The growth medium consisted of MEM supplemented with 10% FCS heated at 56°C for 30 min, bicarbonate, 1% glutamine, and antibiotics (penicillin, 75 U/ml; streptomycin, 75 μg/ml).

HLA Determination. HLA determinations on human PBL and on HeLa cells were performed in the laboratory of Paul Terasaki, UCLA Medical School, Los Angeles, Calif., and by Michele Pellegrino, Department of Molecular Immunology, Scripps Clinic and Research Foundation, La Jolla, Calif.

Antisera, Purification of Human Ig and of Fab'$_2$ Fragments from Rabbit Anti-Human IgG. Human IgG obtained from Pentex Inc., Kankakee, Ill. was chromatographed through a DEAE-cellulose column equilibrated with 0.175 M phosphate-buffered saline (PBS), pH 7.8 (13). Rabbits were immunized at 3-wk intervals with 1 mg of this IgG in Freund's complete adjuvant for the first inoculation, while all subsequent immunizations were done with Freund's incomplete adjuvant. After 3 mo of immunization, rabbits were bled several times, and sera containing more than 1 mg of specific antibody against human IgG/ml were pooled. In order to purify the Fab'$_2$ fragment from this anti-human IgG, 250 ml of the rabbit-pooled antisera heated for 45 min at 56°C was passed through a 100 ml Sepharose 6B column to which more than 200 mg of human IgG had been coupled (34). After extensive washing with 1 M NaCl, and 20% sucrose in 0.1 M NaCl, specific rabbit IgG against human IgG was eluted with 1 M acetic acid in 0.5 M NaCl, neutralized immediately, concentrated, digested with pepsin (14), and rechromatographed through a Sephadex G-200 column. Fab'$_2$ from normal rabbit IgG was purified by chromatography through a DEAE-cellulose column equilibrated with 0.175 M PBS, pH 7.2, and digested with pepsin (13). To obtain rabbit antibody to vaccinia virus, two rabbits were immunized subcutaneously in multiple locations with

5×10^7 PFU of vaccinia virus at 1-wk intervals for a month and then bled. The resulting antisera were heat inactivated (56°C, 45 min). The IgG fraction was purified by DEAE-cellulose chromatography, absorbed on HeLa cell monolayers, and conjugated with fluorescein isothiocyanate as reported (10).

Rabbit ORBC antiserum was raised by immunizing rabbits with increasing amounts of 10% ORBC in PBS (0.5–3 ml) twice weekly for a month. The hemagglutinating titer of the IgG anti-ORBC and its specificity were determined by published immunochemical tests (30).

Human IgG was purified from three human sera harvested from the volunteers before vaccination with vaccinia virus (M. O.) and 15 days after vaccination (M. O. and J. H.). The purified fractions (10 mg/ml PBS) were shown to be free of contaminants by both Ouchterlony and immunoelectrophoresis analyses. IgG from a subject (M. O.) who was immune to vaccinia virus was absorbed by incubating 3 ml of 10 mg immune IgG/ml PBS for 1 h at 37°C and then for 2 h at 4°C with a confluent monolayer of L 929 cells grown in a T75 Falcon flask (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) and previously infected with vaccinia virus. The procedure was repeated twice, and a control was established by absorbing 3 ml of the same IgG preparation with uninfected L 929 monolayers. The IgG preparations were centrifuged for 1 h at 40,000 rpm using a SW40 rotor in a L 65 ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.) to eliminate IgG aggregates, cell debris, virus-antibody complexes, and free vaccinia virus.

Detection of Viral Antigens on Cells. Living fibroblasts infected with vaccinia virus and uninfected were assayed for the presence of surface viral antigens by direct immunofluorescence using fluoresceinated rabbit IgG anti-vaccinia virus. Procedures, equipment used, and cell handling have been described (33). Similar procedures and equipment were used for the detection of membrane-bound immunoglobulins on lymphocytes.

Cytolytic ^{51}Cr-Release Assay. Targets included human fibroblasts, HeLa, or L 929 cells. Cells were grown as monolayers in Falcon T75 plastic flasks, trypsinized, washed twice in growth medium, and dispersed in solution. 100-μl samples containing $1–2 \times 10^4$ cells and 4 μCi ^{51}Cr were added to each well of 6 mm flat-bottomed Falcon TC 3040 microtest plates. After 10 h incubation at 37°C a confluent monolayer containing 1×10^4 or 2×10^4 fibroblasts and 2×10^4 HeLa or L 929 cells formed. The monolayers were then washed twice and incubated with either 50 μl of growth media containing $4–8 \times 10^4$ PFU of vaccinia or 50 μl of growth media for 4 h. After an additional wash the medium was removed and one drop (30 μl) of growth medium was added to each well followed by the addition of immune reagents or growth medium. When anti-vaccinia antibodies or rabbit Fab'_2 anti-human IgG and lymphocytes were used simultaneously, a measured amount of antibody or rabbit Fab'_2 diluted in 100 μl of growth media was added first and allowed to react with the target cells for 30 min before the addition of lymphocytes in 100 μl volume. The final volume was adjusted to 350 μl by the addition of growth medium. When cells infected with measles virus were used, the cells were labeled with ^{51}Cr (4 μCi/well) for 2 h and washed twice. Then the target cells were infected at a MOI of 3, incubated overnight, and finally washed twice before use.

The addition of 350 μl of water and 5 μl of 5% Nonidet P-40 (NP-40) (used as maximal release) released 80–90% of the total radioactivity. 100-μl samples were removed at 6 and/or 18 h and counted in tubes in a Searle Automatic Gamma Counter (Searle Analytic Inc., Des Plaines, Ill.). All the determinations were run at least in triplicate. Variability between triplicate samples never exceeded 10% and averaged 4% of the total sample count. The percent of ^{51}Cr released was calculated according to the following formula: $[(E-S)/(MAX-S)] \times 100$, where E is the ^{51}Cr released from infected target cells in the presence of PBL and anti-viral antibody (13, 35); S is the ^{51}Cr released from infected or uninfected cells in medium or in the presence of anti-viral antibody or other reagents but in the absence of PBL (the higher value was used for calculations); and MAX is the total ^{51}Cr released from infected target cells upon addition of water and NP-40. ^{51}Cr release was calculated separately in each condition for both infected and uninfected cells, and the release induced by PBL or lymphocyte subpopulations from uninfected target cells was subtracted from the values obtained with infected target cells.

Results

Preliminary Experiments. To determine optimal conditions for virus expression on the cell surface, human fibroblasts of different HLA specificities were infected with vaccinia virus at MOIs of 0.1, 1, 2, 4, 8, and 16. The living

fibroblasts, harvested and incubated with fluoresceinated rabbit antibody to vaccinia virus, expressed viral antigens on their surface by 1 h after infection, and by 4 h more than 95% of the fibroblasts were strongly positive when infected at an MOI of 2 or greater. 4 h after infection with an MOI of 0.1, results were inconsistent with between 1 and 10% of cells expressing viral antigens on their surfaces, whereas at an MOI of 1 approximately 50% of the cells were positive (42, 51, and 59% in three different experiments). Uninfected fibroblasts did not express vaccinia virus antigens. HeLa and murine L 929 cells responded to this infection similar to the fibroblasts.

To obtain the most accurate measurements of cell lysis, three different fibroblast lines at varying concentrations were settled into flat-bottomed wells and labeled 12 h later with ^{51}Cr. Vaccinia virus was added at MOIs of 2, 4, 8, and 16, and the spontaneous release for virus-infected and uninfected cells was determined. In an 18 h assay, it was found that the spontaneous release was minimal at MOIs of 2 or 4 (22 ± 3.4%, 24.5 ± 4.1%, and 21.3 ± 2.1%, respectively, for the three different infected target cells at a MOI of 4) and that between 0.5×10^4 and 4×10^4 target cells per well functioned adequately. Spontaneous release of ^{51}Cr from uninfected and virus-infected cells was approximately the same. In subsequent experiments the number of fibroblasts used was $1–2 \times 10^4$ per well and the MOI 4.

Reports conflict as to whether normal human PBL lyse autologous or homologous human skin fibroblasts (36, 37). Therefore, we initially studied the activity of PBL and T or T-depleted (PBL depleted of SRBC-rosetting lymphocytes) lymphocyte subpopulations from three individuals before vaccination against autologous or homologous fibroblasts infected or not with vaccinia at different lymphocyte to target cell ratios. As seen in Table I, all PBL populations lysed both autologous and homologous fibroblasts, infected or not, at a 25:1 lymphocyte to target cell ratio. On occasion, greater lytic activity was detected against uninfected fibroblasts than against infected cells (data not shown). Fig. 1 shows that lysis of uninfected fibroblasts was minimal with T cells and with PBL depleted of Fc receptor-bearing cells. (Similar results were obtained with infected fibroblasts and PBL from persons who were not revaccinated.) In contrast, lysis was maximal with T-depleted lymphocytes (Fig. 1). In all results the amount of immune specific ^{51}Cr release was subsequently expressed after subtraction of the value obtained on uninfected fibroblasts.

Cytotoxicity Induced by PBL after Vaccinia Virus Revaccination

TIME-COURSE AND EFFECT OF LYMPHOCYTE TO TARGET RATIO IN AN AUTOLOGOUS SYSTEM. Fig. 2 shows the percentage of ^{51}Cr specifically released from vaccinia-infected autologous fibroblasts by immune PBL from three volunteers. PBL were harvested from the donors at various intervals after vaccination and added to fibroblasts, which were then assayed for ^{51}Cr release over several time points. ^{51}Cr release was usually maximal around day 7 and decreased markedly by day 11. Immune PBL of five other vaccinated individuals, not shown, produced similar degrees of cytotoxicity with similar time-courses and kinetics. PBL of three individuals were also assayed 21 and 30 days after vaccination, and the specific ^{51}Cr release from infected target cells was always less than 10% at a lymphocyte target cell ratio of 25:1. Generally, ^{51}Cr release was not increased at

TABLE I

*Lysis of Human Fibroblasts Infected with Vaccinia Virus by Different PBL Populations Obtained from Nonrevaccinated Humans**

	Donor	% Specific ^{51}Cr release from human fibroblast from donor (HLA-A/B):					
		L. P. (1, -/8, 37)		M. O. (2, 26/12, 38)		M. D. (2, 3/7, 17)	
		Infected	Uninfected	Infected	Uninfected	Infected	Uninfected
L. P.	Total PBL (1, -/8, 37)	27‡	28	25	26	28	29
	T cells	20	21	22	19	21	23
	T-depleted PBL	32	31	29	29	32	33
M. O.	Total PBL (2, 26/12, 38)	26	27	24	26	27	29
	T cells	20	18	23	20	21	23
	T-depleted PBL	29	28	29	30	31	33
M. D.	Total PBL (2, 3/7, 17)	30	31	27	29	31	32
	T cells	22	21	22	21	21	22
	T-depleted PBL	35	33	30	31	35	35
Spontaneous release		18	17	20	18	19	18

* Total PBL, T, and T-depleted PBL (total PBL depleted of lymphocytes which rosette with SRBC treated with neuraminidase) from nonrevaccinated subjects were added to virus-infected or uninfected fibroblasts at an effector to target cell ratio of 25:1. Amount of ^{51}Cr specifically released after 18 h incubation was determined (see Materials and Methods).

‡ Number reflects the ^{51}Cr released without correction as a mean of triplicate determinations. The standard error of the mean (SEM) did not exceed 6%.

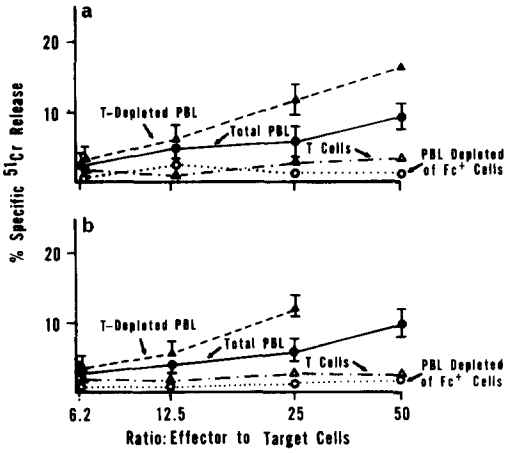

FIG. 1. Total PBL, T cells, T-depleted PBL, and PBL depleted of lymphocytes bearing Fc receptors from a nonrevaccinated subject were added to uninfected autologous (*a*) or homologous (*b*) fibroblasts at different effector to target cell ratios. The amount of ^{51}Cr released was determined after 18 h incubation, calculated as described in the Materials and Methods, and expressed as the mean of triplicate determinations ± SEM. Similar values (not reported) were obtained by using infected fibroblasts as targets and the same lymphocyte population. Spontaneous release was 23 ± 3% for fibroblasts in (*a*) and 20 ± 5% for fibroblasts in (*b*).

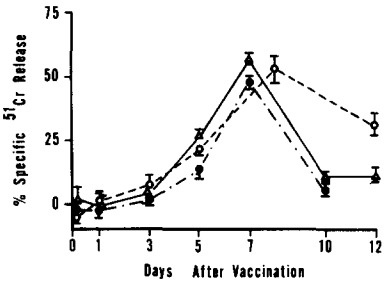

FIG. 2. Three donors' total PBL harvested at different days after vaccination with vaccinia virus were tested against autologous fibroblasts infected or not with vaccinia virus at an effector to target ratio of 25:1. The amount of ^{51}Cr released was determined after 18 h incubation, calculated as described in the Materials and Methods, and expressed in the figure for infected fibroblasts after subtraction of values obtained for uninfected fibroblasts. (Mean of triplicate determination ± SEM.) Spontaneous release for uninfected fibroblasts was between 18 and 25%, while for infected fibroblasts it was between 19 and 26%.

day 7 as compared to day 0 when PBL were added to uninfected fibroblasts, but when enhanced, this increase never exceeded 7% of the total ^{51}Cr release.

CYTOTOXICITY INDUCED BY PBL IN AUTOLOGOUS, HOMOLOGOUS, OR HETEROLOGOUS COMBINATIONS. Immune PBL from 10 donors of known HLA specificities were tested 7 days after vaccination against a panel of fibroblasts of known HLA specificities and against murine L 929 cells. There was no relationship between the homology of HLA antigens of PBL and infected target cells and the ability of immune PBL to lyse vaccinia-infected target cells in 9 of 10 individuals tested (Table II). The one exception was J. H. (Table II, Fig. 3*c*), whose PBL lysed his own fibroblasts and two other fibroblast lines which shared one or two HLA specificities with his PBL. In contrast his PBL did not lyse a third infected fibroblast line devoid of common HLA determinants. Table II shows also that PBL from the three vaccinated females (L. E., P. B., and T. T.) were less cytotoxic for virus-infected targets (always less than 25% at 12.5:1 ratio) than PBL from males. This occurred despite the similarity in response to vaccination by females and males.

Not only did autologous effector and target cell combinations show similar cytolytic activity to homologous combinations, but immune PBL also lysed heterologous L 929 virus-infected cells (Table II). To exclude the possibility that this response was quantitative rather than qualitative, different PBL to target ratios were used in systems sharing or not sharing HLA determinants and in heterologous systems using mouse L 929 target cells. As seen in Fig. 3, homology in HLA antigens between PBL and the virus-infected target cells for individuals R. Z. (Fig. 3*a*) and L. P. (Fig. 3*b*) bears no direct relationship to the degree of lysis measured. Yet, for J. H. (Fig. 3*c*) a quantitative effect occurred; at a high lymphocyte to target cell ratio (20:1), target cells with or without HLA antigens in common with the PBL were lysed to similar degrees, but at lower ratios (5:1) a restriction was seen. PBL from R. Z. (Fig. 3*a*) and L. P. (Fig. 3*b*) lysed L 929 cells and human fibroblasts with a similar efficiency; unfortunately, there were insufficient PBL from J. H. (Fig. 3*c*) to test against L 929 targets.

CELL POPULATIONS INVOLVED IN THE CYTOTOXIC ACTIVITY. Since the killing of virus-infected cells by human immune PBL did not usually require sharing of

TABLE II

*Relationships between HLA Determinants on PBL and Those Expressed on Vaccinia Virus-Infected Targets during Immune Specific Lysis**

Donors	HLA type	% Specific ^{51}Cr release from infected human fibroblasts from donors (HLA-A/B)							Mouse L-929
		J. H. (2, 28/12, 14)	L. P. (1, -/8, 37)	R. Z. (2, 3/35, 8)	T. T. (3, 10/7, -)	M. O. (2, 26/ 12, 38)	M. B. (9, 11/35, -)	C. C. (2, 28/7, 14)	
J. H.	2, 28/12, 14	42.3‡	3.5			39.0		18	
L. P.	1, -/8, 37		45.0	30.5		74.0			51.0
R. Z.	2, 3/35, 8	25.8	27.9	38.1	19.3	28.7		25.8	55.6
T. T.	3, 10/7, -		17.8	18.9	22.9			23.4	
M. O.	2, 26/12, 38	35.5	42.1			33.2	37.4		35.5
M. B.	9, 11/35, -			30.2	38.3	37.3	32.9		36.3
M. D.	2, 3/7, 17	32.6	39.6	39.5	26.8	41.5		32.6	23.0
F. T.	25, 28/12, 39	6.9	11.3	16.5	14.3	13.9			17.5
L. E.	30, 33/14, 13	3.9	7.8	3.2	-3.0	4.3			
P. B.	3, -/7, 5		21.2		7.6	22.9			16.3

* PBL harvested 7 days after vaccination with vaccinia virus were mixed with vaccinia-infected or uninfected fibroblasts of different HLA specificities or with murine L 929 cells at an effector to target cell ratio of 12.5:1 for 18 h. Tests were run in triplicate, and the SEM did not exceed 6%. Experiments were done on different days and the spontaneous release varied from 17 to 29% for uninfected fibroblasts and from 19 to 32% for infected fibroblasts. Within each experiment the spontaneous release was similar among the various fibroblast targets. Spontaneous release for uninfected and infected L 929 cells was between 17 and 26%.

‡ Number refers to the mean value of the specific ^{51}Cr released from infected cells. Autologous combinations are underlined.

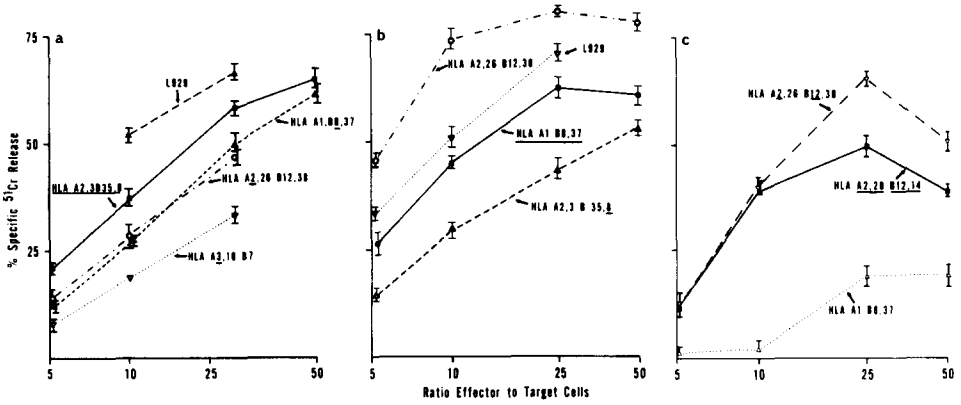

FIG. 3. Three donors' total PBL harvested 7 days after vaccination with vaccinia virus were tested against autologous and homologous fibroblasts as well as murine L 929 cells infected or not with vaccinia virus at different effector to target cell ratio. Condition of the ^{51}Cr assay and calculations are the same as described in Fig. 2. HLA specificities are indicated in the figure and shared HLA specificities between donor PBL and target cells are underlined. (*a*) R. Z., (b) L. P., and (*c*) J. H. Spontaneous release of the various targets was the same as described in Table II.

HLA markers between lymphocytes and infected targets, we sought to identify the killer cells. Whole PBL, T-cell, and non-T-cell subpopulations separately were assayed against autologous, homologous, and heterologous virus-infected target cells. As seen in Fig. 4, the T-depleted subpopulation usually showed one-half to two times greater specific lysis of virus-infected cells than did the total PBL population and was efficient against autologous (Fig. 4*a*) and homologous (Figs. 4*b* and *c*) infected targets. In contrast, T cells caused only limited specific lysis that was also unrelated to homology of HLA antigens between T lymphocytes and target cells.

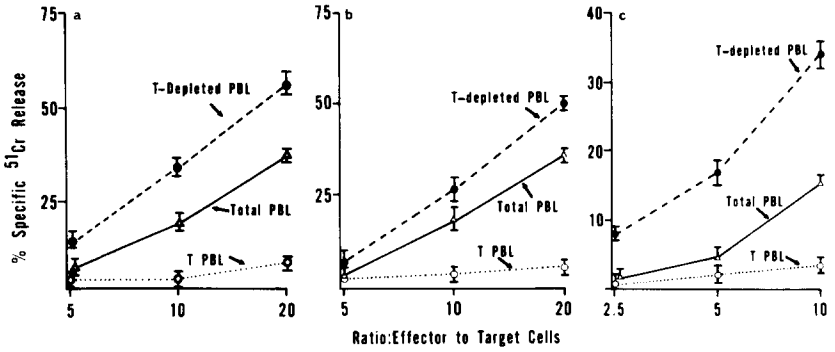

FIG. 4. Two donors' total PBL, T cells, and T-depleted cells harvested 7 days after vaccination with vaccinia virus were tested against autologous (*a*) and homologous (*b* and *c*) targets. Conditions of the ^{51}Cr assay and calculations are the same as described in Fig. 2. Spontaneous release was 18 and 24% for (*a*), 17 and 25% for (*b*) and (*c*) for uninfected and infected fibroblasts, respectively. Donor PBL: (*a* and *b*) L. P.; (*c*) M. D.

TABLE III

*Immune Lysis of Vaccinia Virus-Infected Target Cells by Different Lymphocyte Populations After Vaccination with Vaccinia Virus**

Donors	Lymphocyte populations	% Specific ^{51}Cr release from infected human fibroblasts by donor PBL harvested at:					
		Day 5		Day 6		Day 7	
		Autologous	Homologous	Autologous	Homologous	Autologous	Homologous
L. P.	Total PBL (1, -/8, 37)	6‡	7	23	19	45	30
	T	8	3	5	4	4	5
	T-depleted PBL	6	7	55	32	67	36
T. T.	Total PBL (3, 10/7, -)	6	6	21	25	23	24
	T	5	3	5	5	6	3
	T-depleted PBL	7	7	37	42	39	45

* PBL autologous or homologous to vaccinia virus-infected fibroblasts were harvested 5, 6, and 7 days after vaccination. Total PBL, T, or T-depleted subpopulations were added to virus-infected or uninfected fibroblasts at an effector to target cell ratio of 12.5:1 for 18 h. Tests were run in triplicate and the SEM did not exceed 6%. Spontaneous release of uninfected and infected fibroblasts was between 17 and 22%.

‡ Number refers to the average value of the specific ^{51}Cr released.

Since in prior experiments (not shown), autologous infected fibroblasts were sometimes lysed to a greater extent than homologous fibroblasts and L 929 cells at day 5, we examined the possibility that the early immune response involves a different mechanism and/or a different cell population. However, this is not the case. Table III shows that specific ^{51}Cr lysis of fibroblasts infected with vaccinia virus by autologous or homologous immune PBL, T cells, and T-depleted PBL occurs at days 5, 6, and 7 with the same pattern.

Macrophages and monocytes are not a prerequisite for the lytic activity detected, since all PBL, T cells, and T-depleted PBL had been depleted of plastic-adherent cells. Moreover, in other experiments, three donors' PBL, whether

TABLE IV

*Role of Fc Receptor-Bearing PBL on the Lysis of Human Fibroblasts Infected with Vaccinia Virus**

Donors	Lymphocyte populations	% ^{51}Cr release from infected human fibroblasts	
		Lymphocytes depleted of Fc+ bearing cells:	
		No	Yes
K. P. (not done)	Total PBL	23 ± 4	5 ± 3‡
	T	6 ± 2	6 ± 2
	T-depleted PBL	39 ± 6	8 ± 3
T. T. (3, 10/7, −)	Total PBL	23 ± 5	4 ± 2
	T	4 ± 3	4 ± 2
	T-depleted PBL	39 ± 5	6 ± 3
M. B. (9, 11/35, −)	Total PBL	32 ± 5	4 ± 3
	T	5 ± 3	4 ± 1
	T-depleted PBL	47 ± 6	7 ± 3

* PBL obtained 7 days after vaccination were separated into total PBL, T, or non-T subpopulations. Various lymphocyte populations were depleted of Fc receptor-bearing IgG cells by rosetting with ORBC-IgG (see Materials and Methods). Lymphocyte populations with and without Fc receptor-bearing cells were added to vaccinia virus-infected or uninfected fibroblasts at a ratio of 12.5:1 for 18 h. Subject two was tested on autologous targets, whereas subjects one and three were assayed on homologous test systems. Assays were run in triplicate. Numbers represent the mean specific ^{51}Cr release ± SEM. Spontaneous release of uninfected and infected target cells was less than 23%.

‡ When Fc receptor-bearing PBL purified from total PBL were tested against the same infected target cells at a ratio of 12.5:1, they induced a specific ^{51}Cr release of 15 ± 3% (K. P.), 14 ± 4% (T. T.), and 17 ± 5% (M. B.).

depleted of adherent cells or not, lysed similar amounts of autologous fibroblasts infected with vaccinia 7 days after the donor's vaccination (less than 10% variation at 25:1 PBL:target ratio).

Depletion of Fc receptor-bearing lymphocytes from the total immune PBL or from non-T-cell subpopulations harvested 7 days after vaccination almost completely abrogated the cytolytic potential of both populations (Table IV and Fig. 5). Yet, when Fc receptor-positive lymphocytes were added to virus-infected targets, minimal amounts of immune specific lysis occurred, suggesting either that a second factor or a second cell population was required or that the Fc receptors were blocked by immune complexes (rabbit IgG anti-ORBC-ORBC antigens).

Suppressor T cells may modulate the response of cytotoxic T cells. In humans, T cells bearing Fc receptors for IgG can act as suppressor T cells in some systems (38).[2] Total T-cell populations or T-cell subpopulations depleted of cells bearing Fc receptors for IgG caused minimal or no killing of virus-infected target cells (Table IV). In contrast, when we depleted T cells bearing Fc receptors for IgG in a known suppressor system, the expected suppression was lost.[2]

[2] Oldstone, M. B. A., A. Tishon, and L. Moretta. 1977. Maternal-fetal interrelationships: active thymus derived suppressor lymphocytes are found in human cord blood. *Nature (Lond.)*. In press.

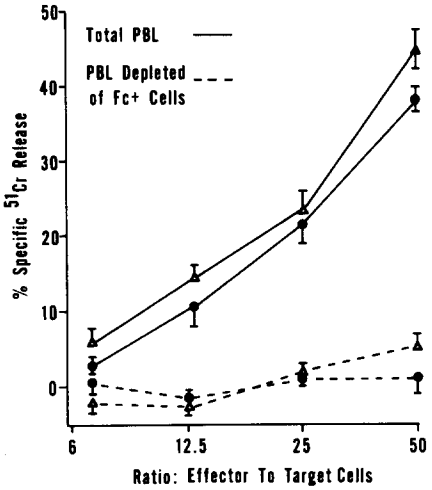

FIG. 5. Two donors' total PBL (●—● and △—△) and total PBL depleted of Fc receptor-bearing lymphocytes (●---● and △---△) harvested 7 days after vaccination with vaccinia virus were tested against human fibroblasts infected or not with vaccinia virus at different effector to target cell ratios. Conditions of the ^{51}Cr assay and calculations are the same as described in Fig. 2. Spontaneous release was 19% for uninfected and 21% for infected fibroblasts.

Inhibition of Lysis of Vaccinia-Infected Fibroblasts. When the FAB'_2 fragment of rabbit anti-human IgG antisera was added in varying amounts to virus-infected target cells before the addition of immune PBL, immune specific lysis decreased more than 95% at 6 μg/ml of the Fab'_2 preparation (Fig. 6). Lysis of uninfected fibroblasts only partially decreased when Fab'_2 rabbit anti-human IgG was added. Rabbit Fab'_2 without antibodies to human IgG did not block immune specific lysis of infected fibroblasts. In other experiments with immune PBL from two additional donors, Fab'_2 anti-human IgG at a concentration of 25 μg/ml similarly abrogated specific lysis of vaccinia virus-infected fibroblasts by more than 95% (ratio 10 PBL:1 target cell).

Specificity of Lysis. Human fibroblasts were infected either with measles virus or vaccinia virus and used as targets to assess the specificity of lysis by immune PBL (Table V). Measles virus-infected fibroblasts were lysed equivalently by PBL harvested at days 0 and 7, whereas vaccinia virus-infected fibroblasts showed a marked increase in ^{51}Cr release when mixed with PBL taken at day 7 compared to PBL obtained at day 0. In other experiments measles-infected fibroblasts were lysed specifically by PBL obtained from children 7 days after vaccination with live attenuated measles virus (L. Perrin, D. Reynolds, and M. B. A. Oldstone, unpublished observation).

Antibody-Dependent Cell Cytotoxicity (ADCC) in the Vaccinia System. The above experiments implicate non-T cells with Fc receptors taken from donors recently vaccinated with vaccinia virus as the responsible immune killer cell. This suggests that ADCC might be the mechanism by which the vaccinia-infected target cells are lysed. Therefore, the ability of nonimmune PBL to lyse vaccinia-infected target cells upon addition of specific antibodies against vaccinia virus was studied. Human IgG preparations purified from the sera of two individuals 15 days after revaccination and one of these two individuals before

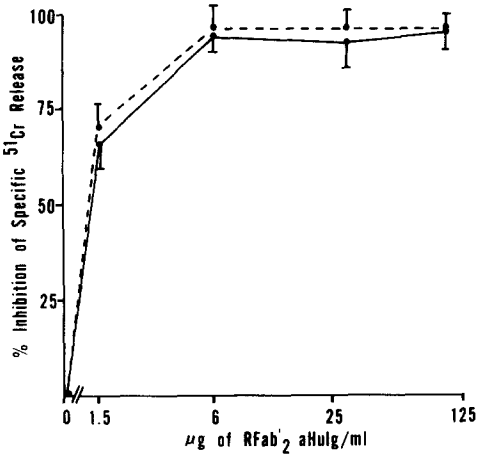

FIG. 6. Different amounts of specific rabbit (R) Fab'_2 anti-human IgG (aHuIg) were added to human fibroblasts infected or not with vaccinia virus before addition of PBL harvested from two individuals 7 days after vaccination with vaccinia virus. Results are expressed as percent of inhibition of lysis obtained in the absence of rabbit Fab'_2 anti-human IgG [42% for donor 1 (M. B.) (●—●) and 26% for donor 2 (T. T.) (●---●)]. The effector to target ratio was 25:1. Conditions of the ^{51}Cr assay and calculations are the same as described in Fig. 2. Spontaneous release was between 21 and 24% for uninfected and infected fibroblasts.

TABLE V

*Specificity of Vaccinia-Immune PBL as Tested on Human Fibroblasts Infected with Vaccinia or Measles Virus**

Donors	% Specific ^{51}Cr release from targets infected with:			
	Vaccinia virus		Measles virus	
	Day 0‡	Day 7	Day 0	Day 7
R. Z. (2, 3/35, 8)	−2 ± 3	55 ± 6	10 ± 3	12 ± 3
T. T. (3, 10/7, −)	−6 ± 2	26 ± 3	5 ± 2	4 ± 2
M. B. (9, 11/35, −)	1 ± 3	41 ± 4	23 ± 5	26 ± 5
Spontaneous release	16–21§	16–20	17–23	19–23

* PBL harvested just before vaccination (day 0) and 7 days after vaccination were added either to vaccinia or measles virus-infected or uninfected target cells at a ratio of 25:1 for 18 h. All three PBL-target systems were autologous and each sample was run in triplicate. Number represents the mean specific ^{51}Cr release ± SEM.

‡ Day of vaccinia virus vaccination.

§ Experiment for each individual was done at a different time. Range of values for spontaneous release of ^{51}Cr from target cells during test periods.

revaccination were tested for their ability to lyse target cells infected or not with vaccinia virus in the presence of nonimmune PBL. None of the three IgG preparations enhanced lysis of uninfected target cells. In contrast, both the postvaccination IgG preparations and to a lesser extent the prevaccination IgG preparation over a wide range of concentrations significantly enhanced lysis of vaccinia-infected target cells (Fig. 7). When absorbed on monolayers of L 929 cells infected with vaccinia virus, the activity of a postvaccination IgG prepara-

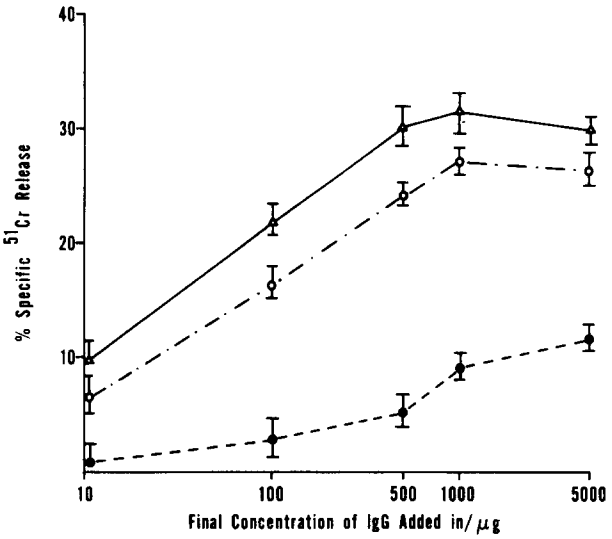

FIG. 7. Three IgG preparations [(△—△), J. H.; (○-·-○), MO after revaccination; (●---●), MO before vaccination] were incubated for 30 min with fibroblasts infected or not with vaccinia virus, before addition of PBL of a nonrevaccinated individual. The effector to target cell ratio was 25:1, and the ^{51}Cr assay read at 6 h. Calculations were the same as described in Fig. 2. The spontaneous release was 9% for uninfected and 10% for infected fibroblasts.

tion decreased by more than 90% (31–2% specific ^{51}Cr release, 0.5 mg/ml IgG, and PBL:target cell ratio of 25:1 in a 6 h assay). Absorption on uninfected L 929 cells had no significant effect (21–18%).

Next, varying PBL to target cell ratios and varying concentrations of immune IgG were studied in the ADCC assay. Fig. 8 shows the effect of varying the PBL:target cell ratios. Maximum lysis occurred using immune IgG at 1 mg/ml and a PBL:target cell ratio of 50:1. At a ratio of 25:1 (ratio used in the previous experiments), the killing of PBL harvested from eight nonrevaccinated donors in the presence and absence of added anti-vaccinia antibody was studied. Table VI shows that specific ^{51}Cr release was marked at 6 h, increased at 18 h when specific anti-vaccinia antibodies and nonimmune PBL were added to infected target cells. This antibody-dependent lysis equalled that caused by immune PBL 7 days after vaccination in the absence of added antibodies (Tables II and V). We also noted that HeLa cells were more readily lysed than human fibroblasts.

To learn whether cytophilic antibodies act as inducers of lysis in the presence of PBL, immune PBL from three individuals bled 7 days after vaccination were treated with trypsin (2.5 mg/ml in MEM at 37°C for 30 min) to remove their IgG, washed twice with complete media, incubated at room temperature for 3 h, washed again, and tested in parallel with untreated immune PBL. Less than 2% immunoglobulin-positive lymphocytes could be detected on the PBL treated by trypsin when tested by direct fluorescence using a polyvalent goat anti-human immunoglobulin serum. Lysis by PBL treated with trypsin was not significantly reduced in two preparations [before/after treatment % specific ^{51}Cr release ±

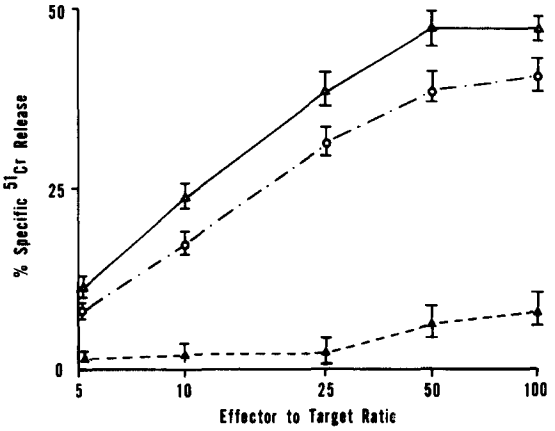

FIG. 8. Total PBL from two individuals not revaccinated: (△---△, ○-·-○) were added at different effector to target ratio to fibroblasts infected or not with vaccinia virus after a previous incubation of the target with 100 μl of a solution containing 1 mg/ml of anti-vaccinia antibodies. (▲---▲) represents the killing of infected target cells by PBL from donor (△---△) in the presence of the same antibody to vaccinia virus previously absorbed with vaccinia-infected L 929 cells (see Materials and Methods). Conditions and calculation as if the ^{51}Cr assay were the same as in Fig. 7. The spontaneous release was 10% for uninfected and 11% for infected fibroblasts.

TABLE VI

*Specific Antibodies to Vaccinia Virus and Nonimmune PBL Lyse Vaccinia Virus-Infected Cells**

Donors	% Specific ^{51}Cr release from infected cells		
	Hu fibroblasts at 6 h	HeLa cells at:	
		6 h	18 h
1	32 ± 5	40 ± 5	46 ± 7
2	20 ± 4	37 ± 4	45 ± 8
3	15 ± 3	24 ± 6	32 ± 3
4	24 ± 6	34 ± 7	57 ± 5
5	12 ± 2	27 ± 3	35 ± 3
6	18 ± 5	28 ± 3	37 ± 6
7	9 ± 7	18 ± 2	22 ± 5
8	24 ± 5	36 ± 3	53 ± 4

* Specific ^{51}Cr release induced by PBL from eight individuals not revaccinated combined with human antibodies against vaccinia virus. Assay was run for 6 h or 18 h and the spontaneous release was 8 ± 3% for human fibroblasts infected for 6 h and 9 ± 3% and 20 ± 5%, respectively, for HeLa cells infected with vaccinia virus. Specific antibodies did not enhance the ^{51}Cr release observed in their absence. Lymphocytes induced a release varying from 5 to 9.5% at 6 h for both infected fibroblasts and HeLa cells, 11-22% at 18 h from infected HeLa cells, and 8-20% from uninfected HeLa cells. These values were subtracted from the values obtained upon addition of anti-vaccinia antibodies to PBL. Results are expressed as the mean of triplicate determination ±SEM.

SEM: 39 ± 5% vs. 35 ± 6% (donor M. B.), 45 ± 4% vs. 36 ± 5% (donor R. Z.)], and reduced 50% in the third [42 ± 6% vs. 21 ± 7% (donor M. O.)].

Discussion

This investigation was undertaken for two purposes. First we wished to follow the reactivity of PBL from humans vaccinated with vaccinia virus against vaccinia-infected human fibroblasts. For ethical reasons, only individuals who had been previously vaccinated during their childhood were revaccinated and all except one developed local induration suggestive of DTH reactions. Second, we wished to study T-cell cytotoxic killing in humans and determine whether an HLA restriction governs killing of virus-infected target by the host's lymphocytes as *H-2* restricts the vaccinia virus T-cell cytotoxicity in the mouse model (26, 27).

A good immune-specific cytotoxic activity against vaccinia-infected cells was detected in PBL by day 5 after vaccination, peaked by days 7–8, and subsided by day 12. There were only weak suggestions of participation of cytotoxic T cells. First, in only 1 of 12 individuals studied was there restricted lytic activity of PBL for fibroblasts sharing common HLA-A or B determinants. Second, a few individuals' PBL lysed more autologous than homologous virus-infected target cells. In contrast, the results indicate that non-T cytotoxic lymphocytes bearing Fc receptors and acting in the presence of specific antibodies are primarily responsible for the lysis of virus-infected target cells.

PBLs from 11 of the 12 individuals newly vaccinated with vaccinia virus lysed fibroblasts infected with vaccinia virus. The lytic activity with one exception was not limited to autologous fibroblasts since homologous fibroblasts with or without HLA antigens of the A and B loci shared by the immune PBL, as well as murine L 929 cells, were also lysed. Thus, histocompatibility between effector and target cells did not seem to be necessary for lysis. These results appear to contrast with experiments reported in the mice whose spleen cells must share at least one gene product of the *K* or *D* region of the *H-2* complex with the virus-infected target cell if the latter is to be lysed. However, since the major effector cells in human PBL after vaccination are non-T cells and they require the participation of antibodies, HLA restriction may not be expected. Other considerations are that the HLA system may be far more polymorphic and present more cross-reactivity than the *H-2* system (39). Also, the HLA antigens are not completely defined; for example, we do not know the HLA determinants of the C locus.

The probability that a non-T cell was the cytotoxic cell in our system is supported in several ways. First, fractionation of human PBL localized the cytolytic activity in the non-T-cell population. Second, removal of Fc receptor-bearing cells from PBL, T-cell, or T-depleted cell populations abolished cytolytic activity. Third, T cells alone could not lyse vaccinia-infected target cells even after the removal of putative T-suppressor cells. If present, blastlike cytotoxic T cells are likely in the T-enriched population, as T blasts rosette with SRBC (40). While removal of lymphocytes bearing Fc receptors abolished the lytic activity, any Fc receptor-bearing T cells by analogy with the murine T cells, are probably not cytotoxic T cells (41).

Two points clearly emphasize both the specificity of the lysis for vaccinia-infected target cells and the development of an active immune response. First, immune PBL induced lysis of vaccinia-infected target cells only 5–7 days after vaccination. Second, cytotoxic activity was increased against vaccinia-infected cells, but not against measles-infected cells, by PBL harvested at 7 days after vaccination as compared to PBL harvested at the day before vaccination (day 0). Similar specificity was observed when PBL obtained 7 days after vaccination with measles virus, significantly enhanced killing of measles virus but not vaccinia virus-infected target cells (L. Perrin, D. Reynolds, and M. B. A. Oldstone, unpublished observations). In contrast, others showed that nonimmune PBL naturally lyse human fibroblasts infected with cytomegalovirus (42) and postulated that this cytolysis could be directed against an early alteration of cell membranes commonly found on several types of human-transformed cells or cells infected with herpes virus (43–45).

The specificity of the lytic process in our system probably emanated from an ADCC process. The effector cell seemed to be a K cell, based on three findings. First, lytic activity was associated with the non-T-cell subpopulation; second, lysis was abolished by removal of Fc receptor-bearing cells from either PBL or non-T cells; and third, Fab'_2 fraction of rabbit anti-human IgG selectively inhibited the lysis of human fibroblasts infected with vaccinia virus by immune PBL. Such inhibition has been demonstrated in other K-cell systems (46, 47), probably because the Fc sites of the bound anti-viral antibody were covered or altered by anti-Fc antibodies.

The anti-viral antibodies needed for lysis in this system could have been carried on the surfaces of the lymphocytes. Alternatively, a more likely hypothesis is that these antibodies could have been formed and released in culture by antibody-producing cells, since trypsin treatment of the immune lymphocytes to remove IgG from their surfaces did not reproducibly suppress cytolysis. If plasma cells secreting specific antibodies are generated after vaccination, this phenomenon would be also transient and could explain the course of the cytolytic activity detected (peak at day 7 and marked decrease at day 11). Moreover, Fc-positive cells without added antibodies showed less activity than the non-T-cell population, suggesting that a second factor was involved in this reaction. Alternatively, the activity of Fc-positive cells could have been blocked by immune complexes (rabbit anti-ORBC-ORBC). Experiments are in progress to clarify these points.

That ADCC could be an efficient mechanism for in vitro lysis of vaccinia-infected cells was shown by incubating target cells infected with vaccinia virus with human anti-vaccinia antibodies, then adding PBL from eight individuals who had not been revaccinated. None of these PBL lysed more vaccinia-infected target cells than uninfected target cells unless anti-vaccinia antibodies were added to the reaction. Furthermore, the activity of the immune IgG preparation could be absorbed with vaccinia-infected but not with uninfected L 929 cells.

Why are we unable to demonstrate cytotoxic T cells in humans after vaccinia virus inoculation, whereas this virus easily raises cytotoxic T cells in mice? The existence of a K-cell-like mechanism need not exclude T-cell killing although antibody may block T-cell killing in the mouse model (48–50). However, since

vaccinia virus produced only low antibody titers in our system, this is an unlikely explanation. Could the secondary response we are studying favor ADCC or complement-mediated antibody lysis of infected cells instead of the expected T-cell-mediated lysis? Again, this is unlikely, since in mice the secondary response against vaccinia virus raises a good T-cell response (L. Perrin and R. M. Zinkernagel, unpublished observations) (25). Perhaps only immature cytotoxic T cells are present in human PBL and prolonged contact with the infected cells is required to assure their maturation and transformation into cytotoxic T cells. This explanation also seems improbable since vaccinia virus replicates in lymphocytes, fulfilling the requirement for contact during their stay in lymph nodes or spleen. Moreover, if this hypothesis is true, cytotoxic T cells would not limit the spread of vaccinia virus infection since during the 18 h or more of contact (duration of the ^{51}Cr test) with the infected cells to develop a lytic potential, the infected cells would have already generated infectious virus and their destruction would be without biological significance. Perhaps cytotoxic T cells are trapped in the lesion containing infected cells expressing vaccinia virus antigen and, therefore, are barely detectable in peripheral blood. However, we recently found that peripheral lymphocytes of mice infected with vaccinia virus are as efficient as spleen cells in killing vaccinia-infected target cells, that T cells are involved in this cytotoxic process and that they are *H-2* restricted.[3]

Perhaps the most reasonable explanation is that humans are vaccinated with less than 10^2 PFU, whereas mice are injected with 10^7 PFU. Furthermore, the routes of immunization are different: scarification vs. intravenous injection. Humans undergo only a local reaction such as adenopathy, but mice, due to their size and the route of immunization, usually experience general involvement of the immune system. Whether or not a low antigenic stimulus and a compartmentalization of the immune response in man could lead to a modulation of the immune response and preferential production of specific antibodies instead of generation of cytotoxic T lymphocytes remains to be determined. In order to test this hypothesis we plan to test immune lymphocytes from humans with systemic virus infection. Alternatively, Fc-bearing killer cells may be a subpopulation of T cells or man may respond in a way different than the mouse. Regardless of the explanation, our data clearly show that after humans are vaccinated with vaccinia virus, "classic" cytotoxic T cells are not readily found in the peripheral blood and that effective and efficient killing of virus-infected targets occurs by PBL bearing Fc receptors, probably in the presence of plasma cells releasing anti-viral antibodies.

Again our data raise the question of the respective importance of humoral and cellular immunity in protection and the recovery from viral infection in vivo. In vaccinia-infected mice either the injection of immune antibodies or the transfer of immune spleen T cells protect nonimmune mice from a lethal challenge with vaccinia virus (22, 23, 51). Also, in mice, neutralizing antibody and cytotoxic T cells appear at a similar time after infection. However, cytotoxic T cells appear to peak earlier and have been shown to possess anti-viral activity (21, 27, 52, 53).

[3] Perrin, L., and M. B. A. Oldstone. Manuscript in preparation.

These adoptive transfer models may not adequately reflect events occurring during a primary or secondary vaccinia infection. The available data for murine vaccinia and ectromelia infections is compatible with the interpretation that cytotoxic T cells are likely mainly involved in the early stages of primary infection whereas antibodies function later and in protection against secondary infections. In man, ADCC is demonstrated in many viral and tumoral systems; however, it has been difficult to find it in similar systems in mice. In contrast, the reverse has been observed for cytotoxic T cells. Whether or not this is related to the experimental conditions used or to the respective importance of these different effector mechanisms in man and in mice remains to be determined. One of the major biological functions attributed to cytotoxic T cells is the lysis of the virus-infected cells before virus assembly of the virus progeny; such an effect has been demonstrated in mice (52, 53) for specific cytotoxic T cells. Similarly, in humans, PBL in the presence of specific antibodies can lyse herpes virus-infected target cells before assembly of viral progeny (54). We have recently made similar observations using measles virus and have further shown that ADCC was efficient long before antibody-mediated complement-dependent lysis of the infected target cells occurred.[3] Therefore, ADCC may be an efficient mechanism in humans in restricting the spread of virus infection, and we suggest that ADCC probably plays an important role in virus infections of humans.

Summary

After vaccinia virus vaccination of human volunteers, local indurations developed within 10 days, and regional adenopathy was detected in half of the individuals. Their peripheral blood lymphocytes (PBL) harvested at different days after vaccination showed specific activity against target cells infected with vaccinia virus with a peak activity at day 7. The specificity of the cytotoxic activity was not related to HLA markers, since autologous, homologous, and heterologous infected target cells were lysed with the same efficiency. The cytotoxic activity was caused by PBL that did not rosette with sheep erythrocytes and could be depleted by more than 90% by removing Fc receptor-bearing cells. T-cell-depleted PBL showed a one-half to two times greater cytotoxicity than intact PBL. The cytotoxic activity could also be abrogated by more than 95% by rabbit Fab'_2 anti-human IgG. On the other hand, nonimmune PBL lysed vaccinia-infected target cells in the presence of specific antibodies against vaccinia virus, thus demonstrating that ADCC could be efficient in lysing vaccinia-infected target cells. We conclude that after vaccination, antibody-forming cells arise and provide specific anti-viral antibody and that the cytotoxic cells detected in this reaction are K cells. These experiments suggest that antibody-dependent cell cytotoxicity may be of major importance in the recovery of man to virus infections.

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References

1. Fenner, F., B. R. McAulan, C. A. Mims, J. Sambrook, and D. O. White. 1974. The Biology of Animal Viruses. Academic Press, Inc., New York.
2. Mims, C. A. 1964. Aspects of the pathogenesis of virus diseases. *Bacteriol. Rev.* 28:30.
3. Oldstone, M. B. A., and F. J. Dixon. 1976. Immunopathology of viral infections. *In* Textbook of Immunopathology. P. Miescher and H. Müller-Eberhard, editors. Grune & Stratton, Inc., New York. 303.
4. McLaren, L. C., J. J. Holland, and J. T. Syverton. 1959. The mammalian cell-virus relationship. I. Attachment of poliovirus to cultivated cells of primate and non primate origin. *J. Exp. Med.* 109:475.
5. Lennette, E. 1964. Diagnostic procedures for viral and rickettsial diseases. *Am. Public Health Assoc. Yearb.* 43.
6. Svehag, S. E. 1968. Formation and dissociation of virus antibody complexes with special reference to the neutralisation process. *Prog. Med. Virol.* 10:1.
7. Murphy, B. R., and L. A. Glascow. 1968. Factors modifying host resistance to viral infection. III. Effect of whole body X-irradiation on experimental encephalomyocarditis virus infection in mice. *J. Exp. Med.* 127:1035.
8. Oldstone, M. B. A. 1975. Virus neutralization and virus induced immune complex disease. *Prog. Med. Virol.* 19:84.
9. Rawles, W., and W. A. Tomkins. 1975. Destruction of virus infected cells by antibody and complement. *In* Viral Immunology and Immunopathology. A. Notkins, editor. Academic Press, Inc., New York. 99.
10. Perrin, L. H., B. S. Joseph, N. R. Cooper, and M. B. A. Oldstone. 1976. Mechanism of injury of virus-infected cells by antiviral antibody and complement: participation of IgG, $F(ab')_2$, and the alternative complement pathway. *J. Exp. Med.* 143:1027.
11. Shore, S. L., C. M. Black, F. M. Melewicz, P. A. Wood, and A. J. Nahmias. 1976. Antibody-dependent cell-mediated cytotoxicity to target cells infected with type 1 and type 2 herpes simplex virus. *J. Immunol.* 116:194.
12. Andersson, T., V. Stejskal, and B. Härfast. 1975. An in vitro method for study of human lymphocyte cytotoxicity against mumps infected target cells. *J. Immunol.* 114:237.
13. Perrin, L. H., A. Tishon, and M. B. A. Oldstone. 1977. Immunologic injury in measles virus infection. III. Presence and characterization of human cytotoxic lymphocytes. *J. Immunol.* 118:282.
14. Kreth, H. W., and V. ter Meulen. 1977. Cell mediated cytotoxicity against measles virus in SSPE. I. Enhancement by antibody. *J. Immunol.* 118:1291.
15. Jenner, E. 1798. An enquiry into the causes and effects of the variolae vacciniae. Sampson-Low, London. 13.
16. Allison, C. A. 1974. Interaction of antibodies, complement components and various cell types in immunity against viruses and pyogeneic bacteria. *Transplant. Rev.* 19:5.
17. Allison, A. C., and W. H. Burns. 1968. Immunogenicity. F. Brock, editor. North-Holland Publishing Co., Amsterdam.
18. Lipsey, A. I., M. J. Kahn, and R. P. Boland. 1967. Pathologic variants of congenital hypogammaglobulinemia: an analysis of 3 patients dying of measles. *Pediatrics.* 39:659.
19. Cooper, M. D., H. P. Chase, J. T. Lowthan, W. Krivit, and R. A. Good. 1968. Wiskott-Aldrich syndrome: an immunologic deficiency involving the afferent limb in immunity. *Am. J. Med.* 44:499.
20. Good, R. A., W. D. Kelly, J. Rotstein, and R. L. Varco. 1962. Immunological deficiency diseases: agammaglobulinemia, hypogammaglobulinemia, Hodgkin's disease and sarcordosis. *Prog. Allergy.* 6:187.

21. Hathaway, W. E., J. H. Githers, W. R. Blackburn, V. Fulginiti, and C. H. Kempe. 1965. Aplastic anemia histiocytosis and erythrodermia in immunologically deficient children. *N. Engl. J. Med.* 273:953.
22. Blanden, R. V. 1974. T cell response to viral and bacterial infection. *Transplant. Rev.* 19:56.
23. Blanden, R. V. 1971. Mechanisms of recovery from a generalized viral infection: mousepox. II. Passive transfer of recovery mechanisms with immune lymphoid cells. *J. Exp. Med.* 133:1074.
24. Gilden, D. H., G. A. Cole, and N. Nathanson. 1972. Immunopathogenesis of acute central nervous system disease produced by lymphocytic choriomeningitis virus. II. Adoptive immunization of virus carriers. *J. Exp. Med.* 135:874.
25. Gardner, I. D., and R. V. Blanden. 1976. The cell mediated immune response to ectromelia virus infection. II. Secondary response in vitro and kinetics of memory T cell production in vivo. *Cell. Immunol.* 22:283.
26. Zinkernagel, R. M., and P. C. Doherty. 1974. Restriction of in vitro T cell mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* (*Lond.*). 24:701.
27. Doherty, P. C., R. V. Blanden, and R. M. Zinkernagel. 1976. Specificity of virus-immune effector T cells for H-2K or H-2D compatible interactions: implications for H-antigen diversity. *Transplant. Rev.* 29:89.
28. Mendelsohn, J., A. Skinner, and S. Kornfeld. 1971. The rapid induction by phytohemagglutinin of increased α-aminoisobutyric acid uptake by lymphocytes. *J. Clin. Invest.* 50:818.
29. Gilbertsen, R. B., and R. S. Metzgar. 1976. Human T and B lymphocyte rosette tests: effect of enzymatic modification of sheep erythrocytes (E) and the specificity of neuraminidase-treated E. *Cell. Immunol.* 24:97.
30. Ferrarini, M., L. Moretta, R. Abrile, and M. L. Durante. 1975. Expression of a receptor for IgM by human T cells in vitro. *Eur. J. Immunol.* 5:70.
31. Zwartow, H. T., J. C. N. Westwood, and G. Appleyard. 1962. Purification of poxviruses by density gradient centrifugation. *J. Gen. Microbiol.* 29:523.
32. Early, E., P. H. Peralter, and K. M. Johnson. 1967. A plaque neutralization method for arboviruses. *Proc. Soc. Exp. Biol. Med.* 125:741.
33. Joseph, B. S., and M. B. A. Oldstone. 1975. Immunologic injury in measles virus infection. II. Suppression of immune injury through antigenic modulation. *J. Exp. Med.* 142:864.
34. Cuatrecasas, P. 1970. Protein purification by affinity chromatography. *J. Biol. Chem.* 245:3059.
35. Brunner, K. T., J. Mauel, J. C. Cerottini, and B. Chapuins. 1968. Quantitative assay of the lytic action of immune lymphoid cells on ^{51}Cr labeled allogeneic target cells in vitro; inhibition by isoantibody and by drugs. *Immunology.* 14:181.
36. Liburd, E. M., V. Pazderka, A. S. Russel, and J. B. Doosetor. 1976. Fibroblast lysis by lymphocytes from normal persons and SLE patients on short-term culture. *Immunology.* 31:767.
37. Parkman, R., and F. S. Rosen. 1976. Identification of a subpopulation of lymphocytes in human peripheral blood cytotoxic to autologous fibroblasts. *J. Exp. Med.* 144:1520.
38. Moretta, L., S. R. Webb, C. E. Grossi, P. M. Lydyard, and M. D. Cooper. 1977. Functional analysis of two human T-cell subpopulations: help and suppression of B-cell responses by T cells bearing receptors for IgM or IgG. *J. Exp. Med.* 146:184.
39. Dupont, B., J. A. Hansen, and E. J. Yunis. 1976. Human mixed lymphocyte culture reaction: genetics, specificity and biological implications. *Adv. Immunol.* 23:108.
40. Politis, G., H. G. Plassara, and H. Thomou-Politi. 1975. DNA present in multilayer

rosette formed by lymphocytes stimulated by phytohaemagglutinin. *Nature* (*Lond.*). 257:485.

41. Pang, T., I. F. C. McKenzie, and R. V. Blanden. 1976. Cooperation between mouse T-cell subpopulations in the cell mediated response to a natural poxvirus pathogen. *Cell. Immunol.* 26:153.
42. Diamond, R. D., R. Keller, L. Gerald, and D. Finkel. 1977. Lysis of cytomegalovirus-infected human fibroblast and transformed human cells by peripheral blood lymphoid cells from normal human donors (39650). *Proc. Soc. Exp. Biol. Med.* 154:259.
43. De Vries, J. E., S. Cornain, and Ph. Rümke. 1974. Cytotoxicity of non-T-versus T-lymphocytes from melanoma patients and healthy donors on short and long-term cultured melanoma cells. *Int. J. Cancer.* 14:427.
44. Peter, H. H., J. Pavie-Fischer, W. H. Fridman, C. Aubert, J. P. Cesarini, R. Rombu, and F. M. Kourilsky. 1975. Cell mediated cytotoxicity in vitro of human lymphocytes against a tissue culture melanoma cell line (IGR3). *J. Immunol.* 115:539.
45. De Vries, J. E., M. Meyering, A. van Dongen, and Ph. Rümke. 1975. The influence of different isolation procedures and the use of target cells from melanoma cell lines and short term cultures of the nonspecific cytotoxic effects of lymphocytes from healthy donors. *Int. J. Cancer.* 15:391.
46. Austin, R. M., and C. A. Daniels. 1976. Inhibition by rheumatoid factor, anti Fc and staphylococcal protein A of antibody-dependent cell-mediated cytolysis against herpes simplex infected cells. *J. Immunol.* 117:602.
47. Trinchieri, G., P. Bauman, M. de Marchi, and Z. Tokes. 1975. Antibody dependent cell mediated cytotoxicity in humans. I. Characterization of the effector cell. *J. Immunol.* 115:249.
48. Welsh, R. M., and M. B. A. Oldstone. 1977. Inhibition of immunologic injury of cultured cells infected with lymphocytic choriomeningitis virus: role of defective interfering virus in regulating viral antigenic expression. *J. Exp. Med.* 145:1449.
49. Germain, R. N., M. E. Dorf, and B. Benacerraf. 1975. Inhibition of T-lymphocyte-mediated tumor-specific lysis by alloantisera directed against the H-2 serological specificities of the tumor. *J. Exp. Med.* 142:1023.
50. Rajan, T. V., B. R. Bloom, and S. G. Nathenson. 1976. Regulatory variants for the expression of H-2 antigens. II. Level of discrimination of cell-mediated cytolysis. *J. Natl. Cancer Inst.* 56:1229.
51. Worthington, M. 1973. Mechanism of recovery from systemic infection with vaccinia virus. II. Effect of x-irradiation and neonatal thymectomy. *J. Infect. Dis.* 127:512.
52. Zinkernagel, R. M., and A. Althage. 1977. Antiviral protection by virus-immune cytotoxic T cells: infected target cells are lysed before infectious virus progeny is assembled. *J. Exp. Med.* 145:644.
53. Jackson, D. C., G. L. Ada, and R. Tha Hla. 1976. Cytotoxic T cells recognize very early minor changes in ectromelia virus infected target cells. *Aust. J. Exp. Biol. Med. Sci.* 54:349.
54. Shore, S. L., T. L. Cromeans, and T. J. Romano. 1962. Immune destruction of virus infected cells early in the infectious cycle. *Nature* (*Lond.*). 262:695.