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T-Cell-Mediated Cytotoxic Response to Mumps Virus in Humans

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Mumps-specific T-cell-mediated cytotoxic activity against virus-treated autologous lymphocytes was studied after peripheral blood lymphocytes of sensitized subjects had been incubated with ultraviolet light-inactivated virus antigen. Generation of the cytotoxic activity in vitro was associated with an antecedent lymphoproliferative response to mumps virus. The virus specificity of the effector cells was demonstrated by a lack of lysis of type 1 parainfluenza virus (HVJ)-treated as well as of type A influenza virus-treated autologous target cells. This activity was largely associated with E-rosette-forming T lymphocytes as revealed by negative selection of a population from cultured whole lymphocytes. In addition, sequential investigations for subjects with a natural mumps virus infection clearly demonstrated individual characteristics of the cytotoxic response. Therefore, the assay described could be used to reflect mumps virus-specific T-cell-mediated immunity in humans.

The response of specific cell-mediated immunity may play an important role in the cellular events which follow certain viral infections of humans and animals (23). Although a number of in vivo and in vitro studies of humans have suggested the induction of mumps virus-specific cell-mediated immunity after exposure to this virus (7, 8, 17-19), the exact nature of the response has not been fully established. Moreover, recent studies on virus-specific T-cell-mediated cytotoxicity in the murine system have demonstrated that the effector cells recognize certain antigens of the major histocompatibility complex (MHC) as self in addition to virus antigen on the target cell surface (12, 25, 26). Although a distinct pattern of this MHC restriction in humans has not been clearly established, the presence of a similar restriction has been suggested by the cytotoxicity of influenza virus-infected human cells (2, 14). This genetically determined immune reaction, therefore, most likely is a universal phenomenon and only a methodology along these lines can provide a reliable tool for evaluating human T-cell-mediated immunity to viral infections. In the present study, we devised a method for in vitro generation of mumps-specific cytotoxic T cells to lyse virus-treated autologous cells by secondary culture of lymphocytes from sensitized subjects; we show that the assay is useful for studying mumps-specific cell-mediated immunity in humans.

MATERIALS AND METHODS

Virus. Concentrated virus antigen was prepared by centrifugation of mumps virus (Enders strain)-infected chick amniotic fluid at 36,000 × *g* for 2 h after the crude host materials had been removed by centrifugation at 4,000 × *g* for 30 min and by resuspending the pellets into phosphate-buffered saline in 1/20 of the original volume. This antigen had 512 to 1,024 hemagglutination units (HAU)/0.025 ml by conventional hemagglutination testing. The control antigen was prepared from uninfected amniotic fluid. The antigen of type 1 parainfluenza virus (HVJ) or type A influenza virus [A/Adachi/2/57(H_2N_2)] grown in allantoic fluid was prepared in a similar manner.

Lymphocyte donors. Heparinized peripheral blood was obtained from subjects in the acute stage of mumps and from immune adults. Normal neonates (cord blood) were also included for study. The method of tissue culture infectivity (5) was used to determine specific neutralizing antibody in the serum of these donors. Lymphocytes were separated from a single blood specimen by the Ficoll-Hypaque fractionation technique (3) and were washed and processed for preparation of both effector cells and target cells as described below.

Preparation of effector lymphocytes. The cells were suspended to 2 × 10^6/ml in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 60 μg of kanamycin sulfate per ml, 20% fetal calf serum, and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer. Five milliliters of the suspension was cultured in no. 3012 plastic flasks (Falcon Plastics, Oxnard, Calif.) in the presence of ultraviolet light (UV)-inactivated mumps virus or of control antigen in an incubator with an atmosphere of 5% CO_2 at 37°C for the indicated number of days. On the day of the cytotoxicity test, the cells were washed and the number of viable cells was determined by trypan blue exclusion.

Target cells. Two milliliters of the lymphocyte suspension (2 × 10^6 cells/ml) was maintained under conditions similar to those described for preparation of the effector cells, with phytohemagglutinin (final

concentration, 10 μg/ml; Wellcome Research Laboratories, Beckenham, England) having been added 4 days before the cytotoxicity test was performed. The phytohemagglutinin-stimulated lymphocytes were washed, and the pellet was incubated with 200 μCi of $Na_2{}^{51}CrO_4$ (The Radiochemical Centre, Amersham, England) and 12,000 HAU of live mumps virus (0.3 to 0.6 ml of the stock virus) at 37°C for 1 h. The cells were again washed extensively, diluted to the appropriate number in RPMI 1640 medium with 10% fetal calf serum, and used as target cells. Regardless of the immune status of the subjects, the target cells prepared in this manner constituted 78 to 83% of cells with virus-specific surface antigen as determined by fluorescein isothiocyanate-labeled anti-mumps virus rabbit antibody (Toshiba Co., Nigata, Japan).

Cytotoxicity assay. The assay was performed by a micromethod as described previously (20). One hundred microliters of the target cell suspension (10^4/well) and the same volume of suspensions of various numbers of effector cells were placed in triplicate in the wells of round-bottomed microculture plates (Linbro Plastics, New Haven, Conn.). The plates were centrifuged at 250 × *g* for 3 min and incubated for 4 h at 37°C with 5% CO_2 in air. One hundred microliters of the supernatant fluid was withdrawn, and the radioactivity was counted in a gamma spectrometer. The percentage of ^{51}Cr release was calculated by the following formula:

$$\text{immune release (\%)} = \frac{\text{release in the presence of effector cells} - \text{spontaneous release at 4 h}}{\text{½ total activity} - \text{spontaneous release at 4 h}} \times 100$$

The difference between the immune release by control effector cells and that of mumps-sensitized cells was regarded as the specific immune release.

Determination of lymphoproliferative activity. The proliferative response of cultured lymphocytes was measured by the uptake of [^{3}H]thymidine. Lymphocytes were suspended, and 100 μl was transferred in triplicate to a microculture plate. Then 0.2 μCi of [^{3}H]thymidine per 20 μl (specific activity, 5.0 mCi/mmol; The Radiochemical Centre) was added, and the culture was incubated for 4 h in 5% CO_2 in air at 37°C. The cells were washed, collected on a glass-fiber filter (Skatronas, Lierbyen, Norway) by a multiple automated cell harvester (Labo Mash; Labo Science, Tokyo, Japan), and processed further for counting by a liquid scintillation counter (15).

Cell separation procedures. The character of the effector cells was studied by negative selection of a subpopulation on the basis of the surface receptor from cultured whole lymphocytes. Depletion of lymphocytes with receptors for sheep erythrocytes (SRBC) (11) was performed by adding 10% neuraminidase (grade B, no. 480717; Calbiochem, San Diego, Calif.)-treated SRBC to the cells and by collecting the nonrosetting cells at the interface of a Ficoll-Hypaque gradient. An analogous procedure was used to deplete cells with receptor for either the Fc fragment of immunoglobulin G (IgG) (EA) or complement (EAC) after cultured lymphocytes were mixed with bovine erythrocytes (BRBC) coated with the IgG fraction of anti-BRBC rabbit serum or with BRBC coated with an IgM fraction of the antiserum and fresh human serum as complement, respectively (16).

RESULTS

Kinetics and dose response of cytotoxic activity. To determine the optimal period for and to investigate the cellular event for in vitro generation of cytotoxic activity, sets of lymphocyte cultures were tested for cell viability, lymphoproliferation, and cytotoxic activity for 9 days at 48-h intervals starting 24 h after initiation of the culture. Representative data from three donors are presented in Fig. 1. Viable lymphocytes, expressed as percentage of the initial cell input, decreased gradually, and only 20 to 30% were viable at the 9th day of culture.

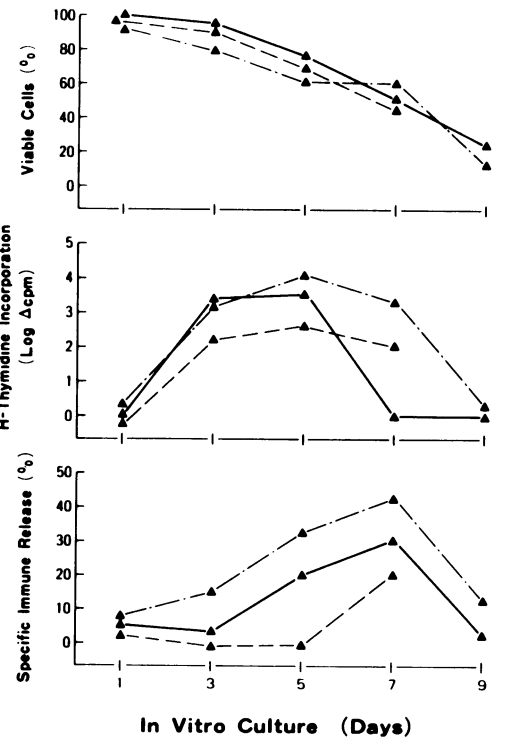

FIG. 1. *Kinetics of viable cells, [^{3}H]thymidine uptake and cytotoxic activity during culture of lymphocytes from three immune adults with 1,024 to 2,048 HAU of UV-inactivated mumps virus. [^{3}H]Thymidine uptake was expressed as log counts per minute in 1/50 of each culture. A target cell-to-effector cell ratio of 1:20 was used for the cytotoxicity test. Target cells at the 1st day of culture were established with phytohemagglutinin 3 days before the initiation of the study as described in the text. Percentage of ^{51}Cr release by effector cells cultured with control antigen was always less than 3% and was subtracted from that of virus-sensitized effector cells.*

Maximal lymphoproliferative activity was observed between the 3rd and 5th days after initiation of the culture, whereas the peak of cytotoxic activity was consistently detected on the 7th day. Thereafter, both lymphoproliferative and cytotoxic activities declined rather rapidly.

The dose response of the cytotoxic activity was evaluated in the next series of experiments, in which lymphocytes from three subjects were cultured for 7 days with various amounts of the virus antigen, i.e., 512 to 4,096 HAU, and were then tested for cytotoxic activity with various target cell-to-effector cell ratios. In general, the responses to different concentrations of the virus antigen increased in a dose-dependent manner, as had been expected. However, it was found that 4,096 HAU of the antigen could induce near-maximal cytotoxic activity, particularly in the high-responding subjects. Therefore, for the purpose of this study, we generally used leukocytes cultured for 7 days with 4,096 HAU of the antigen in subsequent experiments.

Virus specificity. The specificity of the effector cells in recognizing viral surface antigens of target cells was determined by using autologous lymphocytes treated with type A influenza virus or HVJ virus. Effector cells cultured with either of these viruses could effectively lyse the cells treated with the corresponding virus (Table 1), suggesting that these target cells are susceptible to virus-specific cell-mediated cytotoxicity. However, mumps virus-sensitized effector cells could not lyse the influenza- or HVJ-infected cells although they had high cytotoxic activity to mumps virus-treated target cells.

Cytotoxic activity in natural mumps infection. The responses in four subjects with a natural mumps virus infection were followed for 3 to 8 weeks after the onset of illness. The sequential changes and the activity obtained from four normal neonates (cord blood) are presented in Fig. 2. No significant cytotoxic activity was detected in cord blood lymphocytes. A variable degree of activity was detected in subjects in the acute phase of infection, and the peak responses were observed during 2 to 4 weeks after the onset of illness. Subsequently, the activity declined promptly in three cases and gradually in the remaining case. Thus, the study clearly demonstrates the individual characteristics in the development of specific cell-mediated immunity after natural infection with mumps virus.

Lymphocyte subpopulation responsible for cytotoxic reaction. Repeated analysis for subpopulations of in vitro-stimulated lymphocytes on the 6th or 7th day of culture revealed that 55 to 60% of the population consisted of

TABLE 1. *Virus specificity of in vitro mumps virus-sensitized effector cells*

Donors no.[a]	Antigen in vitro[b]	Target/effector cell ratio	% Immune release from virus-infected and uninfected autologous cells[c]			
			A/Adachi	HVJ	Mumps	Uninfected
1	A/Adachi	1:40	16.3	NT[d]	3.3	1.1
		1:20	17.6	NT	1.2	2.1
	HVJ	1:40	NT	21.3	5.5	5.1
		1:20	NT	14.1	2.3	1.7
	Control	1:40	−0.9	−7.0	−1.8	3.3
2	Mumps	1:40	NT	−3.0	37.3	−2.1
		1:20	NT	−2.4	40.7	−1.6
3	Mumps	1:40	NT	0.8	34.9	0.2
		1:20	NT	−0.5	32.0	−0.6
	Control	1:40	NT	−3.5	0.1	−0.6
4	Mumps	1:40	2.5	NT	52.8	NT
		1:20	1.8	NT	46.7	NT
	Control	1:40	−0.5	NT	2.2	NT
5	Mumps	1:40	4.1	NT	47.1	NT
		1:20	3.8	NT	42.4	NT
	Control	1:40	6.6	NT	6.7	NT

[a] Donor 1 was an adult immune to mumps virus, and the remaining subjects were in the acute stage of mumps.
[b] 4,096 HAU of UV-inactivated virus antigen was used for all cultures tested.
[c] HVJ virus-treated target cells were prepared in a manner similar to that used for mumps virus-treated target cells. Influenza (A/Adachi) virus-treated target cells were prepared as described previously (2).
[d] NT, Not tested.

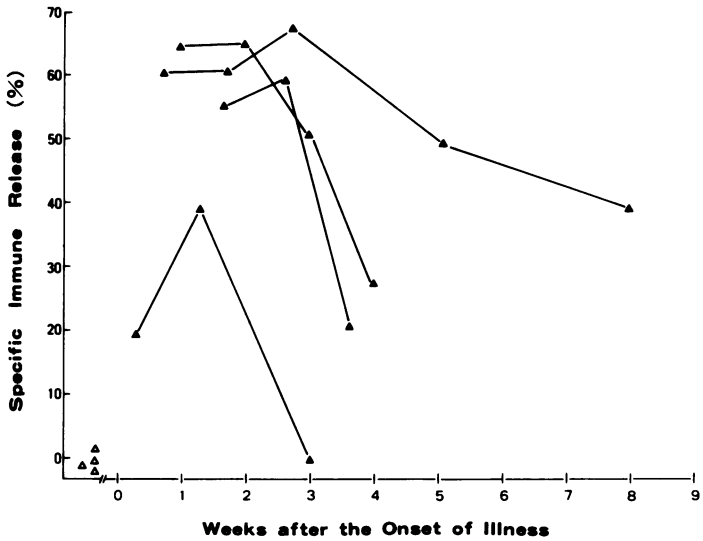

FIG. 2. *Development of mumps virus-specific cytotoxic response in four subjects with natural mumps virus infection (▲) and the activity in cord blood (△). A target cell-to-effector cell ratio of 1:20 was used uniformly.*

lymphocytes forming rosettes with SRBC, fewer than 3% consisted of EA- or EAC-rosette-forming cells, and the remainder consisted of blastoid cells, some of which had low but detectable affinity for SRBC. Although these observations suggested that T cells were involved in this cytotoxicity, additional experiments were performed to confirm this role of T cells. The cytotoxic activity of cells depleted of EA- or EAC-rosette-forming cells was similar to that of the untreated control lymphocyte population (Fig. 3). In contrast, a depletion of E-rosette-forming cells reduced the activity to one-third to one-sixth of the control activity. Therefore, it was obvious that the cytotoxicity was largely T-cell mediated.

DISCUSSION

T-cell-mediated immunolysis of virus-infected cells requires that the target cells express both viral surface antigen and a part of the MHC-coded products which are identical to those on effector cells (12, 25, 26). Recently, it has been also shown that syngeneic cells exposed to inactivated Sendai virus or its isolated envelope are susceptible to virus-specific immunolysis (13) and that the fusion glycoprotein of the virus envelope has an essential role in formation of the antigen on virus-coated target cells (9, 21). Therefore, treatment of human lymphocytes with mumps virus, which is also known to have fusion activity, could provide susceptible target cells for T-cell-mediated immunolysis.

Very few studies have defined the in vitro characteristics of the generation of virus-specific human cytotoxic lymphocytes. The kinetics of culture with mumps virus suggest that the lymphoproliferative response of a sensitized clone is required for the secondary generation of cytotoxic activity. Furthermore, changes in the proportions of the lymphocyte subpopulations based on their surface characteristics were observed. In particular, an increase of blastoid cells, which have low affinity for SRBC, was frequently detected at the 6th or 7th day of culture, in agreement with the observations of Biddison et al. (2). However, our results were somewhat different from those of Wright and Levy (24) in that they detected Fc receptor-possessing cytotoxic cells with restricted allogeneic specificity after lymphocytes of the donor were incubated with measles virus-infected autologous or allogeneic fibroblasts. In our system, fewer than 3% of the cells had Fc receptor, with no significant decrease in cytotoxic activity being observed after depletion of the cells. Although these observations seem to suggest that the nature of the resultant effector cells may be greatly influenced by the mode of antigenic presentation to the precursor cells during in vitro culture, the precise mechanisms involved remain to be elucidated.

The specificity of cytotoxic lymphocytes in recognizing various antigenic components on virus-infected target cells has not been well established. Recently, Braciale (4) demonstrated the presence of a cross-reactive clone which can recognize type A-specific antigen in the response of mice to type A influenza viruses. It has also

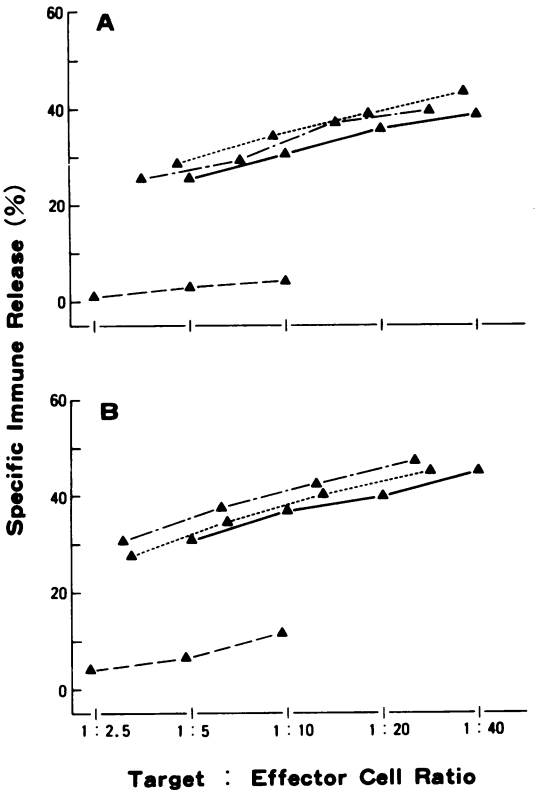

FIG. 3. *Effects of depletion of a lymphocyte subpopulation on the cytotoxic reaction of two subjects in the acute stage of mumps. The activity after depletion of E (---)-, EA (---)-, or EAC (-----)-rosette-forming cells was compared with that of the untreated whole lymphocytes (——). Contamination of E-rosette-forming cells in the E-depleted population was 0 to 12%, with no EA- or EAC-rosette-forming cells being detected after depletion of corresponding cells.*

been shown that the human secondary cytotoxic T-cell responses to type A influenza virus in vitro are predominantly directed against such cross-reactive determinants (2). However, in the present study effector cells generated in secondary response to mumps virus did not react with autologous target cells treated with HVJ, suggesting either specificity of the effector cells to mumps virus-specific antigen or the lack of such a cross-reactive determinant at the surface of the target cells. Therefore, the majority of the effector cells were seemed to be directed to the mumps virus-specific antigen of the target cells.

Since a cellular response mediated by virus-specific cytotoxic T cells had been suspected to play an important role in the pathogenesis of viral infection, efforts had been focused on obtaining in vitro correlates of immunity in humans. In response to mumps, xenogeneic (1, 10) or allogeneic (6) target cells have been used thus far for this purpose. Although a study of animals (22) has suggested the possible detection of virus-specific cytotoxic activity by allogeneic combination of effector and target cells, methods used previously do not seem to be appropriate for quantitative estimation of mumps-specific T-cell-mediated immunity in humans.

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