EVIDENCE THAT CYTOTOXIC T CELLS ARE PART OF THE HOST'S RESPONSE TO INFLUENZA PNEUMONIA

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Cytotoxic T-cell responses to influenza infection in mice (1–6) and after immunization with intact viral vaccine (7) have been described. This response to infection has been noted in splenic tissue as well as in draining cervical lymph nodes (2). Those studies indicated that the specificity of the T-cell response was restricted by the histocompatibility (H-2) antigen and the viral hemagglutinin (1, 2, 7). The purpose of the present experiments was to extend these observations by evaluating whether: (*a*) cytotoxic T cells were present in the peripheral blood of infected animals; (*b*) cytotoxic T cells were present in the lungs of mice with pneumonia, and (*c*) there was a relationship between the number of lymphocytes and cytotoxic T cells in the lungs and the peripheral circulation that might indicate a contribution of cytotoxic T lymphocytes to the pathology of influenza pneumonia.

Materials and Methods

Mice. BALB/c mice (H-2^d) and C3H mice (H-2^k) were obtained from the Animal Production Unit of the National Institutes of Health, Bethesda, Md. Mice of the congeneic strains B10.D2 and B10.BR which express the H-2^d and H-2^k haplotypes, respectively, were provided from The Jackson Laboratory, Bar Harbor, Maine. The H-2 type of these mice was confirmed using monospecific anti-H-2 typing antisera.

Viruses. A/England/42/72 (H3N2) and B/Hong Kong/5/72 viruses were obtained from Harold Kaye of the Center for Disease Control, Atlanta, Ga. A/Port Chalmers/1/73 (H3N2) virus (MRC-9) was obtained, after recombination with A/PR/8/34 virus, from Geoffrey Schild of the National Institute of Biological Standards and Control, Hampstead, England. Each of these viral strains was adapted for growth in mice by repeated intranasal passage of infected lung tissue. Stocks of viruses for use in immunization were prepared by inoculating fertile hens' eggs. The allantoic fluid harvests from these eggs were used as the source of virus in the experiments reported in this paper. The identity of the virus pools was confirmed serologically using monospecific chicken anti-influenza typing antisera.

Infection and Virus Titration. 3-wk-old BALB/c and C3H mice were inoculated intranasally with 0.2 LD_{50} of the above virus strains. Control mice received diluent intranasally. Infection was verified by observing pulmonary pathology, by the development of specific anti-influenza antibodies, or by assay of pulmonary virus titers. Lungs were homogenized and then diluted in phosphate-buffered saline (PBS)[1] containing 0.1% bovine serum albumin. Three eggs were inoculated intra-allantoically with each 10-fold dilution. After 48 h incubation at 35°C, the allantoic fluids were tested for the presence of viral hemagglutinin with a 0.25% suspension of chick erythrocytes in PBS.

[1] *Abbreviations used in this paper:* EID_{50} dose infections for 50% of eggs inoculated; FCS, fetal calf serum; MEM, minimal essential medium; PBS, phosphate-buffered saline; SR, stimulation ratio; LD_{50}, dose lethal for 50% of inoculated mice.

Preparation of Spleen Cells. Spleens were removed from infected and control mice at various times after viral inoculation. Erythrocytes were lysed by exposure of the spleen cell suspensions to ammonium chloride buffer (8). After centrifugation the lymphocytes were suspended in minimal essential medium (MEM) supplemented with 10% heat inactivated fetal calf serum (FCS) at a concentration of 10^7 viable cells/ml.

Preparation of Cervical Lymphocytes. Lymph nodes were removed from the anterior cervical region of control and infected mice and were finely teased apart on a Petri dish using a surgical scalpel. After gentle pipetting in MEM supplemented with 10% FCS the lymphocytes were washed twice, counted, and resuspended at a concentration of 10^7 viable cells/ml.

Preparation of Lung Lymphocytes. Lungs were surgically removed and freed of extraneous tissue. The lungs were then minced with scissors, and pressed through a sterile 60 mesh stainless steel screen with a glass pestle and 10 ml of MEM supplemented with 10% FCS per lung. Lymphocyte separation was accomplished on a Ficoll Hypaque gradient as described by Boyum (9). The cells were then washed twice in MEM, supplemented with 10% FCS, counted, and resuspended at a concentration of 10^7 viable cells/ml.

Preparation of Peripheral Blood Lymphocytes. Lymphocytes in heparinized (40 U/ml; Hynson, Westcott and Dunning, Inc., Baltimore, Md.) blood drawn from the heart were also isolated on Ficoll Hypaque gradients, washed, and suspended as described for the lung lymphocytes.

Removal of T Lymphocytes. Lymphocytes from immune and control mice were treated for 30 min at 37°C with a 1:10 dilution of an AKR antiserum against C3H-θ (anti-θ serum), washed, and exposed to a 1:10 dilution of guinea pig serum as a source of complement. This treatment killed approximately 40% of the spleen cell population and specifically eliminated viable T cells. Lymphocytes incubated with goat serum against mouse immunoglobulin and with complement or in medium with complement were similarly tested for residual cytotoxicity. T-cell enriched lymphocytes were prepared by passage through a nylon wool column according to the method of Julius et al. (10). Immunoglobulin bearing, bone marrow-derived lymphocytes (B cells) selectively adhere to these columns, and the effluent contains a highly-enriched population of T cells.

Preparation of Target Cells. Kidneys were removed from BALB/c, B10.BR, and B10.D2 weanling mice and primary cultures were established. Confluent monolayers of cells in 35-mm plastic Petri dishes were inoculated with approximately $10^{6.5}$ 50% egg-infectious doses (EID_{50}). The dishes were incubated at 36°C for 1 h, washed, and incubated in fresh medium for an additional 18 h. At this time greater than 90% of the cells showed membrane-associated influenza antigen when stained by the fluorescent antibody technique (see below). The cells were removed from the dishes by trypsinization and, after washing, were incubated with ^{51}Cr ($Na_2{}^{51}CrO_4$, New England Nuclear Corp., Boston, Mass.) at a concentration of 100 μCi/ml for 30 min at 36°C. The cells were washed twice in medium and resuspended to a concentration of 10^5/ml.

Lymphocyte Cytotoxicity Assay. ^{51}Cr-labeled target cells (10^4 in a 0.1-ml vol of MEM containing 10% FCS) were added to the wells of a microtiter plate (Microtest II, Bio Quest, BBL & Falcon Products, Becton, Dickinson & Co., Cockeysville, Md.). Replicates of eight wells received 10^6 viable untreated or treated lymphocytes from either infected or uninfected mice in a 0.1-ml vol. Additional wells contained target cells plus 0.1 ml of a 10% solution of Brij detergent (Sigma Chemical Co., St. Louis, Mo.) to determine the maximum releasable ^{51}Cr. The microtiter plates were incubated at 36°C for 18 h. The cells were centrifuged at 1,500 rpm for 10 min. 0.1-ml aliquots were removed from each well and the amount of radioactivity present was determined. The mean value of the counts released by the lymphocytes from infected mice (immune) was compared with both the mean counts released by lymphocytes from uninfected mice (control) and the mean counts released by exposure to Brij detergent (max). The percent specific immune lysis was calculated as follows:

$$\% \text{ lysis} = \frac{\text{immune} - \text{control}}{\text{max} - \text{control}} \times 100.$$

The standard error of the percent specific immune lysis was approximated from the variance of the immune values (11). In each experiment the immune and control values were compared using Student's t test.

Lymphocyte Proliferation Assay. Lymphocytes were resuspended to a concentration of 2×10^6 viable cells per ml in RPMI-1640 medium (Media Unit, NIH) containing 5% FCS and 2×10^{-4} M 2-mercaptoethanol. 0.2-ml vol of lymphocyte suspension from either infected or control uninfected mice were added to wells of microtiter plates (Microtest II, Falcon Plastics). Ultraviolet-inactivated virus or concanavalin A (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N.J.) was added in a 10-μl vol to quadruplicate samples of normal and immune spleen cells. The mcrotiter plates were incubated for 68 h at 37°C in a 5% CO_2:95% air atmosphere. At this time, 1 μCi of [^{3}H]thymidine (6.7 μCi/mmol) (New England Nuclear Corp.) was added per well. 4 h later the cells were collected onto glass fiber filter strips using a MASH II harvester (Microbiological Associates, Walkersville, Md.) and the incorporation of [^{3}H]thymidine determined by scintillation counting. A stimulation ratio (SR) was calculated by dividing the mean cpm incorporated in four replicate cultures containing virus (or mitogen) by the mean cpm of four replicate cultures not containing virus. In all experimental groups, the range of the cpm observed in quadruplicate samples was within 10% of the mean value.

Fluorescent Antibody Staining. The thoracic and cervical tissues were removed in toto. To prevent the lung tissue from collapsing during cryostat cutting, it was instilled through the trachea with embedding medium for frozen tissue specimens (Tissue-Tek II O.C.T. Compound, Miles Laboratories, Inc., Miles Research Products, Elkhart, Ind.). Then the tissues were frozen in isopentane at −70°C. 7-μm thick cryostat sections were fixed in acetone and stained by the indirect fluorescent antibody technique. The primary serum was a hyperimmune mouse serum against the A/Port Chalmers strain (HI titer 1:256) diluted 1:4 in PBS pH 7.4. The conjugate was swine serum globulin against mouse IgG labeled with fluorescein isothiocyanate. Controls for specificity included: (*a*) staining of noninfected lung tissue; and (*b*) substituting normal mouse serum for immune serum.

Results

The mean body weights of infected mice were significantly less than uninfected control mice on days 3 when statistically compared by Student's *t* test for nonpaired data. ($t = 4.4$, $P < 0.001$) and 7 ($t = 3.6$, $P < 0.001$). The mean weight of the lungs on day 7 was significantly higher in the infected mice (280 mg) than in the control mice (220 mg) ($t = 4.47$, $P < 0.001$). The number of lymphocytes in the peripheral blood of infected animals by day 3 was not significantly different from the number of circulating lymphocytes in controls, but there was a significant increase in the number of lymphocytes in the pulmonary tissues (Fig. 1). This increase peaked on day 7 when pneumonia was most marked. There was a threefold increase in the number of lymphocytes isolated from the lung of the infected animals, and simultaneously a 50% drop in the number of circulating peripheral lymphocytes. In the next 2 wk the number of pulmonary lymphocytes gradually decreased and the number of circulating lymphocytes increased as the pneumonia resolved. By day 20 the number of lymphocytes in the lung was almost back to base-line values.

We stained pulmonary tissue from infected and control animals using the fluorescent antibody technique to evaluate whether viral antigens were being expressed in the pulmonary tissue when pathology in the lung and the number of lymphocytes in the lung were increasing. Fig. 2 reveals that 48 h after intranasal administration of live virus, foci of morphologically intact alveolar cells contained viral antigens (Fig. 2 B). This is at the time when early lymphocytic infiltration was present by microscopic examination. By day 7, viral antigen was widespread and most of the alveolar and bronchial epithelial cells were infected and showed advanced degeneration (Fig. 2 C). As expected, only scattered foci of antigen were seen by day 13 when pulmonary virus was no longer isolated from the pulmonary tissue (Fig. 2 D) and no foci were seen by day 21.

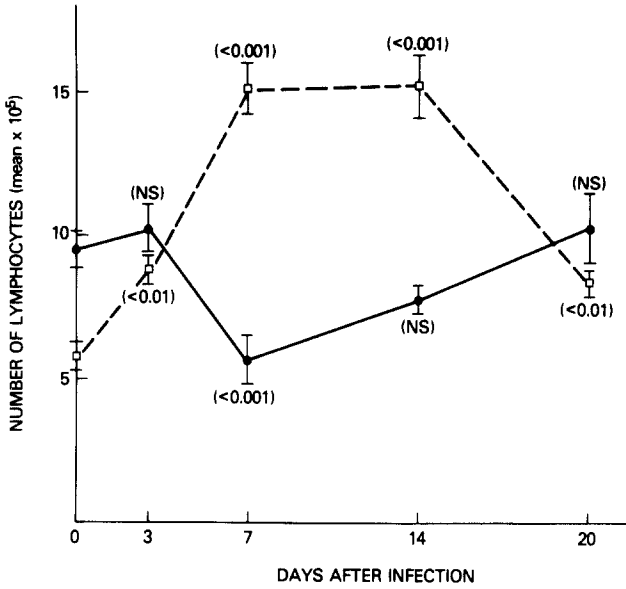

FIG. 1. Mean number of lymphocytes in the peripheral blood (●) and lungs (□) of 10 mice at each interval during the course of infection with A/Port Chalmers virus. Statistical significance was determined by Student's *t* test in comparison with the counts obtained on day 0, with *P* values indicated in parentheses.

Peripheral blood and lungs were evaluated for cytotoxic T-cell activity during the period of influenza infection. Fig. 3 summarizes the results of a typical experiment. Viral replication occurred in the lung and reached a peak by 3 days after infection, then decreased by day 7 and by day 13 no infectious virus was detected. Lymphocytes obtained from peripheral blood, spleen, lung, and cervical lymph node tissues produced significant levels of specific immune lysis as early as day 3. The percent specific immune lysis peaked at day 7 reaching high levels with lymphocytes from draining local lymph nodes, the lungs, the spleens, and the blood of the infected animals.

We next determined the type of lymphocytes in the lung, lymph nodes, and peripheral blood which was causing the cytotoxicity. We used techniques we previously had employed to demonstrate that the splenic and cervical lymph node cytotoxic cells were T cells (1) and the results are shown in Table I. After passing lymphocytes from the lung, lymph nodes, and blood through a nylon-wool column to enrich for T cells, an increase in specific immune lysis occurred. These cells were no longer cytotoxic after treatment with anti-θ serum and complement.

The H-2 specificity of the cytotoxic T lymphocytes in the lung was evaluated. Table II shows that only the target cells which had the same H-2 histocompatibility antigens in the K and D regions as the lymphocyte donors were lysed specifically. The congeneic mice, B10.BR ($H\text{-}2^k$) which are identical to B10.D2 ($H\text{-}2^d$) except for having different determinants in the K and D portion of the H-2 region, were not lysed. Similarly, lymphocytes from the lungs of the infected C3H mice ($H\text{-}2^k$) were able to lyse target cells of B10.BR mice which have the same $H\text{-}2^k$ histocompatibility antigen but not B10.D2 ($H\text{-}2^d$) infected target cells.

We also evaluated the antigenic specificity requirements between the virus used to

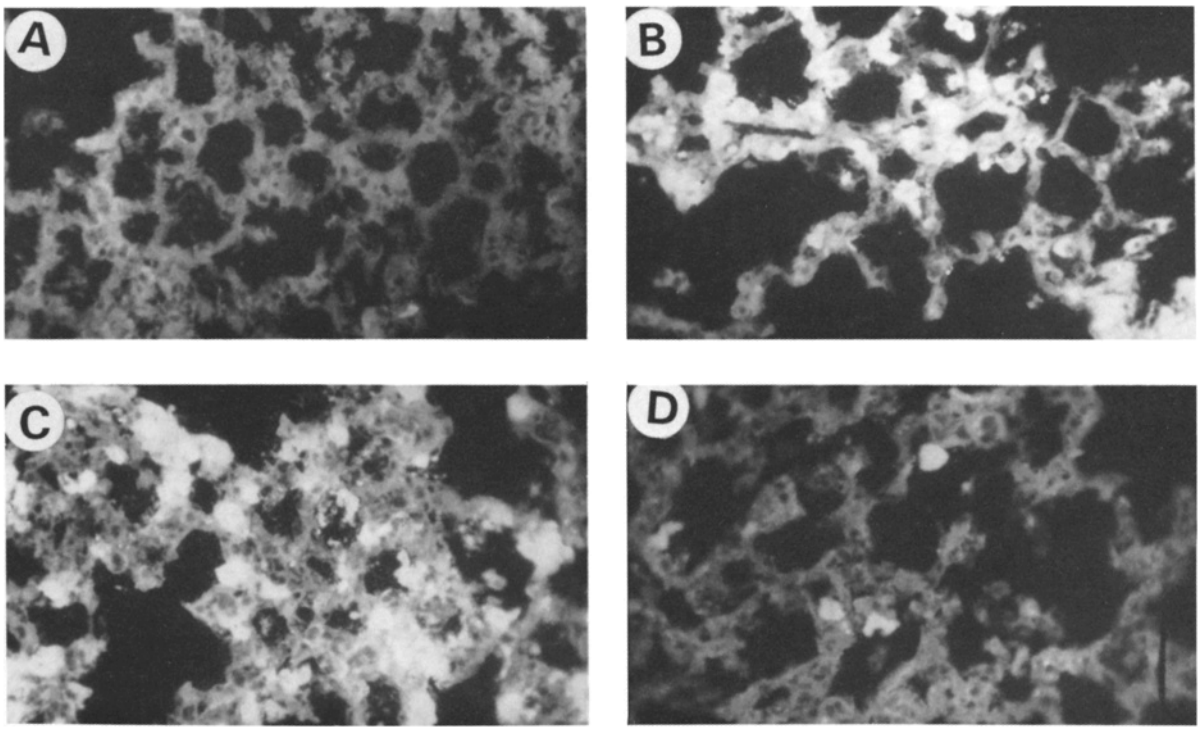

FIG. 2. Lungs of mice infected with mouse-adapted A/Port Chalmers/1/73 (H3N2) virus. A. Control noninfected lung. B. Lung 2 days after infection with many influenza-infected alveolar cells. C. Lung 7 days after infection showing widespread involvement of lung parenchyma. D. Lung 13 days after infection with few scattered influenza-positive cells. Fluorescent antibody staining. All magnifications, × 250.

infect the mice and the virus infecting the target cells. Mice were infected with two influenza A viruses, A/England/42/72, and A/Port Chalmers/1/73. Both of these viruses are members of the H3N2 subtype but they differ somewhat in their H3 hemagglutinin antigen. In addition, mice were infected with the B/Hong Kong virus which has none of the antigenic characteristics of the other two A viruses. 7 days later pulmonary lymphocytes obtained from the mice infected with these virus strains were added to infected target cells. We observed, as shown in Table III, a significant degree of specific immune lysis in each instance in which the same virus was used to infect the mice and the target cells. There was no cross reactivity between the animals infected with A viruses on target cells infected with B/Hong Kong and vice versa. There was, as expected, some degree of cross-reactive lysis observed between the very closely related 1972 and 1973 H3N2 strains but the homologous virus always induced a higher degree of cytotoxicity. These results indicate that cytotoxic T cells in the lungs of mice with influenza pneumonia have a high degree of specificity for the viral hemagglutinin, a finding which we made previously on splenic and cervical lymph node cells of mice infected with influenza (2).

We also measured a proliferative response in lymphocytes obtained from the same mice we had measured the cytotoxic T-cell response. Fig. 4 indicates that a significant proliferative response was detected in lymphocytes of the cervical lymph nodes, spleen,

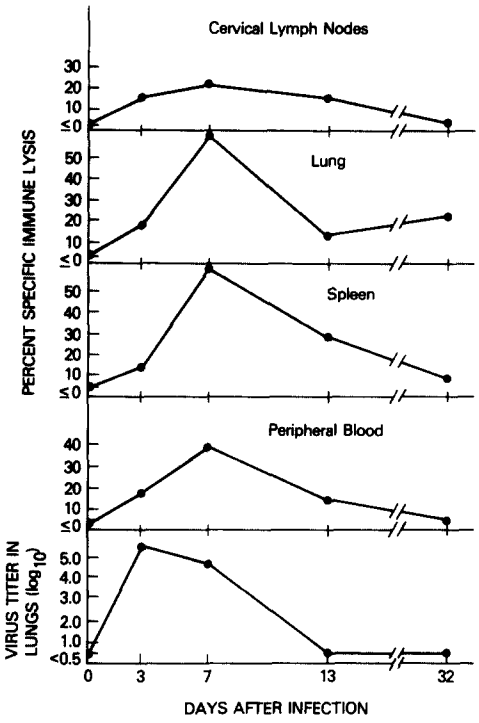

FIG. 3. Pulmonary virus titers and percent specific immune lysis produced by lymphocytes from cervical lymph nodes, lung, spleen, and peripheral blood obtained on days 0, 3, 7, 13, and 32 after infecting mice with 0.2 LD_{50} of A/Port Chalmers virus.

TABLE I

T-Cell-Mediated Cytotoxicity of Influenza-Infected Target Cells

Treatment of lymphocytes from immunized mice	% Specific immune lysis ± SE		
	Lung	Lymph node	Peripheral blood
Exp. 1			
None	22.3 ± 4.2*	15.3 ± 3.3*	32.7 ± 3.4‡
Nylon column nonadherent cells	66.1 ± 4.6‡	22.3 ± 3.4§	37.8 ± 3.4‡
Complement	ND	12.0 ± 3.3*	22.3 ± 3.3‡
Anti-θ plus complement	ND	1.0 ± 3.3	4.8 ± 3.2
Exp. 2			
None	19.1 ± 6.7*	ND	ND
Complement	13.4 ± 6.7	ND	ND
Anti-θ plus complement	−10.0 ± 6.5	ND	ND

Significance was determined by Student's *t* test.
* $P < 0.05$.
‡ $P < 0.001$.
§ $P < 0.01$.

and peripheral blood by day 7. Lymphocytes taken from the lung at the same time did not proliferate in the presence of antigen but did on day 13. This proliferative response has been shown to be T-cell dependent and is more long lived than the cytotoxic T-cell response (12).

TABLE II

H-2 Specificity of Cytotoxic T Cells From Influenza-Infected Mice

Lung lymphocyte donors	% Specific immune lysis ± SE on target cells from		
	BALB/c (H-2^d)	B10.D2 (H-2^d)	B10.BR (H-2^k)
BALB/c (H-2^d)	62 ± 8.2*	30 ± 2.3*	3.9 ± 1.7
C3H (H-2^k)	−17 ± 6.3	−4 ± 2.4	11.3 ± 1.6‡

Significance was determined by Student's *t* test.
* $P < 0.001$.
‡ $P < 0.05$.

TABLE III

Antigenic Specificity of Cytotoxic T Lung Lymphocytes in Influenza-Infected Mice

Virus used to infect mice	% Specific immune lysis ± SE of target cells infected with		
	A/England	A/Port Chalmers	B/Hong Kong
A/England/42/72 (H3N2)	57.2 ± 8.3*	14.5 ± 7.8	−0.3 ± 4.5
A/Port Chalmers/1/73 (H3N2)	42.2 ± 8.2*	21.2 ± 7.9‡	2.7 ± 4.6
B/Hong Kong/5/72	17.8 ± 8.0	5.1 ± 7.8	33.6 ± 4.8*

Significance was determined by Student's *t* test.
* $P < 0.001$.
‡ $P < 0.01$.

Discussion

These experiments were designed to evaluate whether cytotoxic T cells play a role in vivo in the pathogenesis of influenza pneumonia. There did not appear to be conclusive data demonstrating that cytotoxic T cells were present in the blood or target organ of viral infected animals. Blanden reported that during ectromelia infection the lymphocytes in the inflammatory liver nodules were probably derived from the circulating pool of lymphocytes; however, he did not characterize the lymphocytes in the inflammatory nodules as being T cells (13). Hapel and Gardner have reported that the cerebral spinal fluid of mice that had been infected intracerebrally with attenuated ectromelia virus contained inflammatory cells, and that the lymphocytes from the cerebrospinal fluid of the mice were cytotoxic to target cells infected with the same virus. They could block the pleocytosis in the cerebrospinal fluid by treatment of mice with anti-θ sera, and anti-θ and complement decreased the cytotoxic affect of the cerebrospinal fluid cells on target cells infected with ectromelia virus which indicated that the cells in the fluid were T cells. They did not evaluate the lymphocytes in the brains or the meningeal tissues of the infected animals, but assumed that the cells in the cerebrospinal fluid were also present in the inflamed nervous tissue (14).

It is difficult to evaluate experimentally the in vivo contribution of cytotoxic lymphocytes in the pathological processes of infection. Our results do indicate, however, that there is a direct and strong correlation between the increase in the number of lymphocytes in the lungs of mice, and the pronounced cytotoxic activity of T cells in the lungs of mice in the course of influenza pneumonia. These cytotoxic cells are H2 restricted and specific for the hemagglutinin of the infecting virus. In

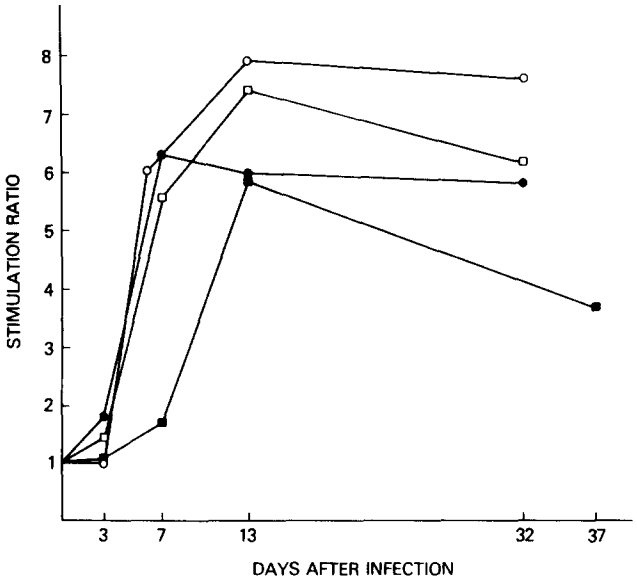

FIG. 4. SR produced by A/Port Chalmers immune splenic (○), cervical lymph node (●), lung (■), and peripheral blood (□) lymphocytes after exposure to 10 μl of ultraviolet-inactivated A/Port Chalmers virus. Lymphocytes were removed from BALB/c mice at various times after intranasal infection of the mice with A/Port Chalmers virus. The results were obtained on pooled populations of lymphocytes from infected and control uninfected mice. A stimulation ratio for each set of cultures was calculated from the mean cpm incorporated in four replicate cultures containing virus divided by the mean cpm incorporated in four replicate cultures not containing virus. In all experimental groups the range of cpm observed in quadruplicate cultures were within 10% of the mean value. The mean [^{3}H]thymidine incorporation in the absence of virus ranged between 5.3×10^2 and 4.6×10^3 cpm for different sets of cultures. Stimulation ratios produced by normal splenic, cervical lymph node, lung, and peripheral blood lymphocytes after exposure to 10 μl of ultraviolet-inactivated A/Port Chalmers virus was less than 1.4.

addition they are present in largest number and have their strongest measurable cytotoxic effect in vitro at a time when the animal has the most advanced degree of pneumonia. This appears to be evidence that cytotoxic T lymphocytes are playing a role in the course of influenza pneumonia. It also appears that influenza infection of the respiratory tract with non-adapted virus induces a cytotoxic T-cell response. We infected mice intranasally with 10^3 EID_{50} of A/Port Chalmers (H3N2) virus which had not been passed in mice, and significant specific immune lysis was detected on day 7 in lymphocytes obtained from the cervical nodes, lung and blood, and on day 14 in splenic lymphocytes. The level of specific immune lysis is lower than that caused by infection with adapted virus but the T-cell response was readily detectable with nonadapted virus, (data not presented). There was also an increase in the number of lymphocytes in the lungs of the infected mice and a corresponding drop in the number of circulating lymphocytes. Thus the cytotoxic T-cell response occurs in influenza infection as well as with more severe influenza disease with pneumonia.

These results are consistent with the hypothesis that precursors of cytotoxic T cells are induced during the course of viral infections and that these sensitized cells are present in lymphoid tissues draining the respiratory tract shortly after infection begins. These T cells could then circulate in the peripheral blood of the infected animal and during random circulation could become concentrated in the tissue, in this case the lung, where there are many cells expressing viral antigens on their surface for which

they have a receptor. After entrapment in the area of the infected target cells, they would be able to directly lyse the target cell, or to elaborate substances such as chemotactic factors which would attract phagocytes, which would help in the lysis and removal of necrotic cells.

In addition to the cytotoxic T-cell response, we also measured the response of another population of lymphocytes in the local lymph node, blood, spleen, and target organ which proliferated when exposed to viral antigen. These cells also probably play a role in the host response to influenza pneumonia.

These experiments appear to strongly support the in vivo contribution of cytotoxic lymphocytes in the acute inflammatory process which is present during influenza pneumonia in mice. Further investigations are required to analyze in more detail the mechanism by which cytotoxic T cells may contribute to the course of influenza pneumonia.

Summary

Cytotoxic T cells were detected in the cervical lymph nodes, lungs, spleen, and peripheral blood of mice with influenza. Lymphocytes decreased in the peripheral circulation and increased in the lung during the period of acute inflammation and pneumonia. Peak cytotoxic T-cell activity was present at the time of marked pulmonary infiltration, and it decreased with resolution of the pneumonia. The cytotoxic T cells in the lung were shown to be H-2 restricted and specific for the hemagglutinin of the infecting virus. The results indicate that hemagglutinin specific cytotoxic T cells are (*a*) induced during influenza infection; (*b*) they circulate in the blood; (*c*) they are present in greatest number; and (*d*) they have their peak cytotoxic effect when pneumonia is most marked. We interpret the results to indicate that specific cytotoxic T cells in the infected target organ are part of the immunological and pathological response to virus infection.

Note added in proof: Experiments recently published by Yap and Ada (*Scand. J. Immunol.* 1978. **7**:73.) report the detection of cytotoxic T cells in the lungs of mice with influenza.

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