

## Cell-Mediated Immune Response to Influenza Virus Infections in Mice

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The local and systemic cell-mediated immune (CMI) responses to influenza virus infection in mice were examined by leukocyte migration inhibition and lymphocyte-mediated cytotoxicity tests. Mice were inoculated intranasally with 5 50% lethal doses of the A/WSN ( $H_0N_1$ ) strain of influenza virus. Cells from the lymph nodes draining the upper and lower respiratory tract were used to measure the local response, and the spleen was the source of cells used for systemic determinations. The local response by pulmonary lymph node cells was greater and appeared earlier than was observed systemically in the spleen. The specificity of the CMI response was investigated by using a heterologous virus strain, A/Jap ( $H_2N_2$ ), and recombinants A/Jap-NWS ( $H_2N_1$ ) and A/NWS-Jap ( $H_0N_2$ ), obtained from a cross between A/Jap ( $H_2N_2$ ) and a virus, A/NWS ( $H_0N_1$ ), with surface antigenic specificity similar to that of the inoculated virus. From the results of both tests used as correlates of CMI, it appeared that the response was specific against the hemagglutinin component of the inoculated virus. No reactivity was observed against the heterologous virus A/Jap ( $H_2N_2$ ) nor against the recombinant A/Jap-NWS ( $H_2N_1$ ) bearing the same neuraminidase as that of the inoculated virus.

There have been a number of reports that a cell-mediated immune (CMI) response against influenza antigens was demonstrable after natural infection and after immunization with killed vaccines (11, 20, 24).

Specifically sensitized lymphocytes may act directly by lysing infected cells (3, 10). Such cytotoxic phenomena may be assayed by the release of radioactive isotope ( $^{51}Cr$ ) from persistently infected target cells by virus-immune lymphoid cells. Cytotoxicity assays of this type have now been studied by using ectromelia (10), measles (14), and lymphochoriomeningitis viruses (3). Although not persistently infected, influenza virus-infected L-cells have all the characteristics required for use as target cells in cytotoxicity experiments. The virus buds from the plasma membrane during maturation, and thus the cell has virus-specific antigen present in its cell membrane.

The leukocyte migration inhibition assay has been used by several workers as a measure of CMI to influenza virus antigens (7, 24).

Recently, Gadol et al. (9) showed that secondary pulmonary and splenic CMI, as measured by the leukocyte migration inhibition assay in animals previously immunized with influenza virus vaccine, appeared 2 to 3 days earlier after a booster dose with the same vaccine. This sug-

gested that pulmonary as well as splenic T-lymphocytes exhibited memory. Thus, the presence of primary and secondary CMI, both systemically and locally in the lung, against influenza virus antigens have been shown experimentally.

The lymphocyte-mediated cytotoxicity assay and the leukocyte migration inhibition test have been widely accepted as correlates of CMI. The purpose of this study was to evaluate the presence and specificity, with respect to the surface antigens of the influenza virus, of the primary local and splenic CMI responses after live virus infection in mice.

### MATERIALS AND METHODS

**Virus strains.** Four strains of influenza virus were used: A/WSN ( $H_0N_1$ ), A/Jap ( $H_2N_2$ ), and two recombinant viruses, A/NWS-Jap ( $H_0N_2$ ) and A/Jap-NWS ( $H_2N_1$ ). The recombinant viruses, which had been isolated from a cross between A/Jap and a virus with antigenic specificity similar to A/WSN, A/NWS ( $H_0N_1$ ), were kindly donated by N. J. Dimmock, University of Warwick, Coventry, England. Virus stocks were grown in the allantoic cavity of 11-day embryonated eggs. Subunit antigen preparations for the *in vitro* leukocyte migration inhibition test were prepared from stock virus by the methods of Webster and Laver (25) and Duxbury et al. (4).

**Mouse strain and inoculation procedure.** Inbred

C3H mice, 6 to 8 weeks of age, were used in the investigation. Mice were inoculated by the intranasal route with 5 50% lethal doses of A/WSN ( $H_0N_1$ ) in 0.025 ml of phosphate-buffered saline (PBS). Age-matched controls received a similar inoculation of diluent.

**Collection of specimens.** At various times post-inoculation, the required animals were each given a lethal dose of sodium pentobarbital (Abbot Laboratories, Sydney, Australia) intraperitoneally.

When the animals were anesthetized but still living, they were bled out from the axilla until death. The spleen, cervical, deep cervical, and mediastinal lymph nodes were removed from each animal and placed in Hanks buffered salt solution supplemented with 10% fetal calf serum (FCS).

The spleens were minced by passing them through a wire mesh, and the erythrocytes were removed by lysis with 0.1844 M  $NH_4Cl$ .

Lymph nodes were pooled and the tissue was teased apart with rat-toothed forceps to release the cells from the lymph node capsule. Tissue fragments were removed by passing the resulting suspension through nylon wool. Spleen and lymph node cells were washed twice in Hanks saline and resuspended to a final concentration of 25% in RPMI 1640 medium supplemented with 10% FCS.

**Leukocyte migration inhibition assay.** Pools of three animals were used for each determination. Sterile siliconized 20- $\mu$ m capillary tubes (Microcaps, Drummond Scientific Co., Broomall, Pa.) were half filled with the cell suspensions, the ends were sealed with a flame, and the tubes were spun at  $100 \times g$  for 3 min. The tubes were cut at the packed-cell interface and portions containing cells were placed, four per dish, in 35-mm Falcon tissue culture dishes (Falcon Plastics, Oxnard, Calif.) each containing 2 ml of RPMI 1640, 0.25 ml of FCS, and 0.25 ml of antigen. Appropriate controls were included. The dishes were placed on a level surface and incubated at 37 C in 5%  $CO_2$  and 95% humidified air for 18 h. The area of migration was quantitated by measuring two diameters, using a microscope fitted with an eyepiece graticule (8).

The effect of antigen on migration of the cells out of the tubes was assessed by calculating the migration, in the presence of antigen, as a percentage of that obtained in its absence (2) using the following formulae:

$$\% \text{ Migration} = 100 \frac{\text{average area of migration with antigen}}{\text{average area of migration without antigen}}$$

$$\% \text{ Inhibition of migration} = 100 \left( 1 - \frac{\text{average area of migration with antigen}}{\text{average area of migration without antigen}} \right)$$

**Lymphocyte-mediated cytotoxicity assay.** (i) **Infection of L-cell monolayers.** The influenza virus strains used to infect L-cell monolayers were the same as those used to prepare antigens for the LMI assay.

The L-cells, which were used as target cells, were grown in 100-mm Falcon tissue culture dishes in

RPMI 1640 and 10% FCS at 37 C, in an atmosphere of 5%  $CO_2$  and 95% humidified air. The culture medium was removed, and the monolayers were washed once with PBS. The cells were infected at a concentration of approximately 5 50% egg infective doses of virus per cell in 1 ml of PBS, and the dishes were incubated at 37 C for 1 h. Excess inoculum was removed and the monolayers were washed once with PBS before the addition of 2.5 ml of RPMI 1640 supplemented with 10% FCS. The cultures were then returned to the incubator until use. Appropriate control cultures, without virus, were treated in parallel. After 5 h of infection with virus, 76% of the L-cells showed positive hemadsorption with chicken erythrocytes. This percentage increased to nearly 100% at 10 h. No cytopathic effect was observed until 26 h postinfection.

(ii) **Labeling of target cells with  $^{51}Cr$ .** Four hours after infection, the L-cell cultures were labeled with  $^{51}Cr$ . Infected cells were trypsinized and transferred into a test tube containing 1 ml of chilled FCS. After washing twice in RPMI 1640, the cells were counted by the trypan blue dye exclusion method, and  $10^6$  viable cells were placed in test tubes, pelleted, and resuspended in a minimal volume of RPMI 1640. They were then incubated with 150  $\mu$ Ci of sodium chromate  $^{51}Cr$  (specific activity, 400 to 600  $\mu$ Ci/mg of chromium, 1 mCi/ml in sterile isotonic saline; The Radiochemical Centre Ltd., Amersham, Bucks., England) per  $10^6$  cells for 30 min at 37 C, with periodic agitation.

The reaction was stopped by the addition of 10 ml of chilled RPMI 1640 to each tube, and the cells were washed twice with medium to remove unincorporated radioactive chromium before being resuspended to give a final concentration of  $10^5$  cells/ml.

(iii) **Experimental procedure.** Appropriate target cells,  $10^4$  in 0.1 ml of medium, were distributed into flat-bottomed wells of Falcon 3040 Microtest II tissue culture plates (Falcon Plastics). Normal or immune spleen and lymph node cells (from pools of two animals for each determination) were then added (in duplicate) to the L-cells in 0.15 ml of medium. The ratio of spleen or lymph node cells to target cells used was 100:1 ( $10^6$  lymphoid cells to  $10^4$  target cells/well). Additional control wells were included, consisting of infected or uninfected L-cells with 0.15 ml of media and serum, instead of lymphoid cells.

After the trays were incubated at 37 C for 18 h in a humidified atmosphere of 5%  $CO_2$  and 95% air, they were spun at  $200 \times g$  for 10 min in an International centrifuge to pellet all cells.

A standard aliquot of supernatant (0.2 ml) was removed from each well and transferred to Packard Auto-Gamma counting tubes and the radioactivity was measured, using a Packard liquid scintillation spectrometer system with a 4022 automatic gamma counter attachment.

The total amount of radioactive chromium available for release from each type of target cell was determined by resuspending the pelleted cells in 0.2 ml of distilled water and transferring them to test tubes containing 1.8 ml of distilled water to lyse the

cells. These test tubes were centrifuged at  $100 \times g$  for 5 min, and the supernatant was transferred to counting tubes. (The amount of chromium released was found to be 80% with a standard deviation of  $\pm 6.5$  of the total counts incorporated into the L-cells.) Chromium release was determined as follows:

$$\% \text{ } ^{51}\text{Cr release} = 100$$

$$\frac{\text{counts in supernatant with lymphocytes} - \text{counts in supernatant with medium}}{\text{counts in supernatant after distilled-water lysis}}$$

**Serum cytotoxicity determinations.** Serum from animals sacrificed as donors of spleen and lymph node cells was diluted 1:2, 1:4, and 1:8 in RPMI 1640 and added to duplicate wells containing  $10^6$  target cells infected with either A/WSN ( $H_0N_1$ ), A/Jap ( $H_2N_2$ ), or uninfected controls, in a total volume of  $0.2 \mu\text{l}$ . The trays were incubated at  $37^\circ\text{C}$  for 30 min, and then 0.5 ml of a 1:2 dilution of guinea pig complement was added to each well and the trays were reincubated for 1 h (10). Appropriate controls were included. The amount of  $^{51}\text{Cr}$  released was determined by the following formula:

$$\% \text{ } ^{51}\text{Cr release} = 100$$

$$\frac{\text{counts in supernatant with serum} - \text{complement counts in supernatant with medium}}{\text{counts in supernatant after distilled-water lysis}}$$

**Infectious virus titer in mouse lungs.** The lungs of two animals were pooled for each day of assay. Mice were inoculated intranasally with 5 50% lethal doses of WSN ( $H_0N_1$ ) and killed by cervical dislocation on days 1 to 7 postinfection. The lungs were removed, placed in PBS, and minced by passing through a wire mesh. This material was centrifuged at  $140 \times g$  for 10 min to remove tissue fragments. The supernatant was made up to 3 ml and filtered through a membrane filter (Millipore Corp.) to remove bacteria and cell debris. The 50% egg-infective dose titer of this fluid was calculated by the method of Fazekas de St. Groth and White (6), and 50% end point of the titrations was determined by the method of Reed and Muench (19).

## RESULTS

**Titer of infectious virus in mouse lungs.** The growth of A/WSN ( $H_0N_1$ ) in the mouse lungs was examined by assaying for infectious virus. Results, expressed as egg-infective units per milliliter of lung extract, are presented in Fig. 1. The titer of infectious virus in the lungs of the animals was maximal at day 5 post-inoculation and subsequently dropped rapidly by day 7.

**Leukocyte migration inhibition.** Inhibition of leukocyte migration was observed in cells from the cervical, deep cervical, and mediastinal lymph nodes and spleens of animals inoculated with A/WSN ( $H_0N_1$ ) against the homologous viral antigens. The response was greater

and appeared earlier in the pulmonary lymph nodes (Fig. 2), maximum mean migration inhibition of 31% and one standard deviation of  $\pm 3.4$  at 8 days, as compared with the spleen response of  $20\% \pm 1.18$  at 13 days (Fig. 3).

The specificity of the responses of lymph node and spleen cells followed similar patterns (Fig. 4 and 5). Inhibition of migration by the recombinant virus containing the same hemaggluti-

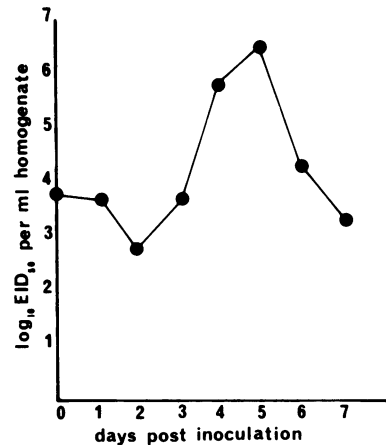


FIG. 1. Titer of infectious virus in the lungs of animals inoculated with 5 50% lethal doses of A/WSN ( $H_0N_1$ ) on day 0. The lungs of two animals were pooled for each determination and results are expressed as the  $\log_{10}$  50% egg-infective dose ( $\text{EID}_{50}$ ) per milliliter of lung homogenate.

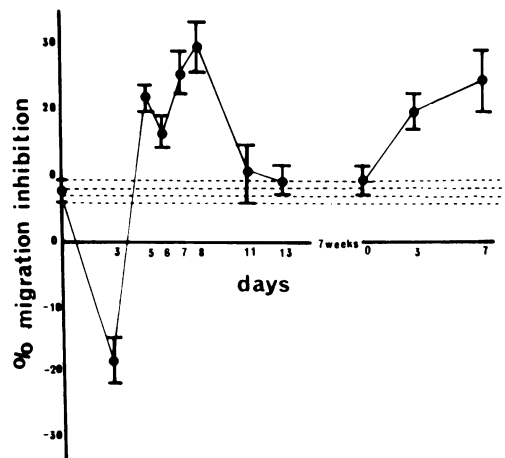


FIG. 2. Inhibition of leukocyte migration. Primary and secondary response of lymph node cells, from animals inoculated intranasally with the A/WSN ( $H_0N_1$ ) strain of influenza virus, against the homologous viral antigens. Variances represent one standard deviation from the mean. Mice were rechallenged 7 weeks later with 1,000 50% lethal doses of the homologous virus, using the same route.

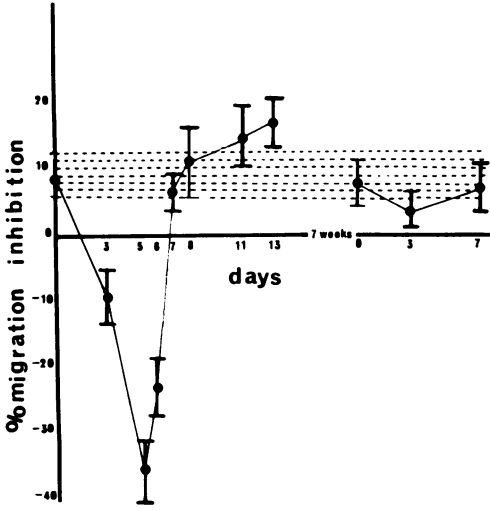


FIG. 3. Inhibition of leukocyte migration. Primary and secondary response of spleen cells, from animals inoculated intranasally with the A/WSN ( $H_0N_1$ ) strain of influenza virus, against the homologous viral antigens. Variances represent one standard deviation from the mean. Mice were rechallenged 7 weeks later with 1,000 50% lethal doses of the homologous virus, using the same route.

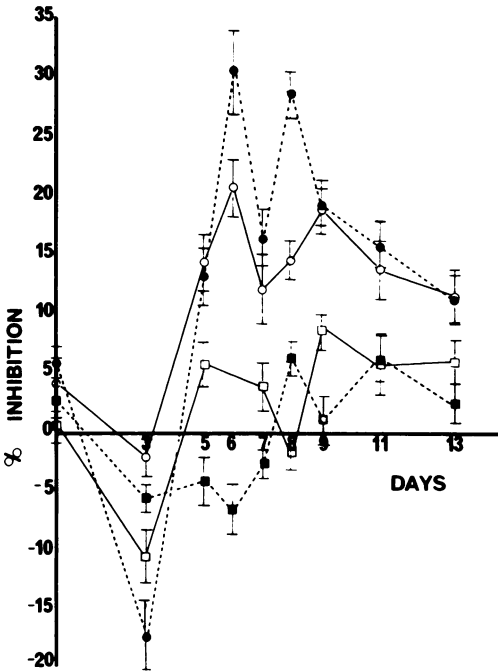


FIG. 4. Inhibition of leukocyte migration. Primary response of lymph node cells from animals inoculated intranasally with A/WSN ( $H_0N_1$ ) on day 0 against antigens prepared from virus strains. Symbols: (●) A/WSN ( $H_0N_1$ ); (■) A/Jap ( $H_2N_2$ ); (○) A/NWS-Jap ( $H_0N_2$ ); (□) A/Jap-NWS ( $H_2N_1$ ). Variances represent one standard deviation from the mean.

nin antigen as that of the inoculated virus showed maximum mean migration inhibition similar to that obtained when the antigen was prepared from the inoculated virus. There was no significant response against the heterologous A/Jap ( $H_2N_2$ ) virus, the surface antigens of which did not cross-react with those of the inoculated virus, nor against recombinant A/Jap-NWS ( $H_2N_1$ ) with the cross-reacting neuraminidase.

When animals were rechallenged with 1,000 50% lethal doses of the A/WSN ( $H_0N_1$ ) strain of influenza virus 9 weeks after primary inoculation, a secondary response was observed locally in the pulmonary lymph nodes (Fig. 2); however, no response was shown by spleen cells (Fig. 3).

Enhancement of migration of cells from lymph nodes and spleen was repeatedly ob-

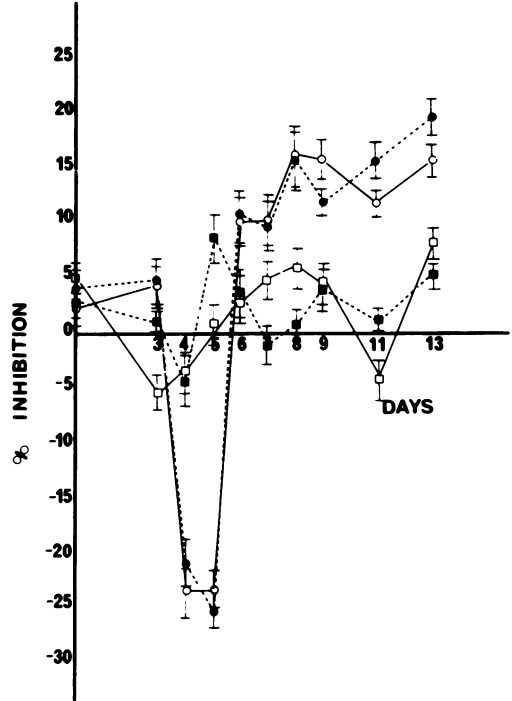


FIG. 5. Inhibition of leukocyte migration. Primary response of spleen cells from animals inoculated intranasally with A/WSN ( $H_0N_1$ ) on day 0 against antigens prepared from virus strains. Symbols: (●) A/WSN ( $H_0N_1$ ); (■) A/Jap ( $H_2N_2$ ); (○) A/NWS-Jap ( $H_0N_2$ ); (□) A/Jap-NWS ( $H_2N_1$ ). Variances represent one standard deviation from the mean.

A/NWS-Jap ( $H_0N_2$ ); (□) A/Jap-NWS ( $H_2N_1$ ). Variances represent one standard deviation from the mean.

served on those days immediately after primary inoculation (Fig. 2-5).

**Lymphocyte-mediated cytotoxicity.** Lymphocytes from lymph nodes of A/WSN ( $H_0N_1$ )-infected animals were specifically cytotoxic for the homologous virus-infected target cells (Fig. 6). This cytotoxicity was significant at day 5 post-inoculation, peaked at day 7, and rapidly decreased to day 20, when no detectable cytotoxicity was observed. The specific immune release of  $^{51}Cr$  by spleen cells from infected animals (Fig. 7) against the homologous virus peaked at day 9 post-inoculation; however, the magnitude of the response, 27.9% with a standard deviation of  $\pm 3.66$ , was considerably less than that observed using lymph node cells ( $52.95\% \pm 5.00$ ).

To determine whether the specific cytotoxicity observed in these experiments could have been mediated by immune serum from the infected animals and complement, serum cytotoxicity assays were carried out. No significant  $^{51}Cr$  release was observed after treatment of the A/WSN ( $H_0N_1$ ) influenza-infected L-cells with serum from animals whose lymphocytes had exhibited cytotoxicity against these target cells.

Spleen and lymph node cells from A/WSN ( $H_0N_1$ )-inoculated animals had little cytotoxic activity against target cells infected by

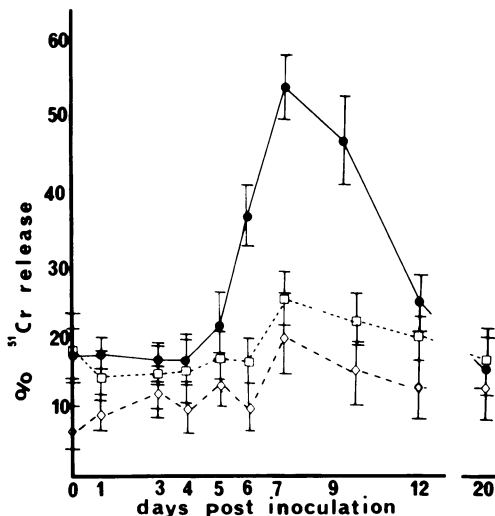


FIG. 6. Percentage of  $^{51}Cr$  release by lymph node cells from animals infected intranasally on day 0 with the A/WSN ( $H_0N_1$ ) strain of influenza virus against the following target cells: (●) A/WSN ( $H_0N_1$ )-infected L-cells; (□) A/Jap ( $H_2N_2$ )-infected L-cells; (◇) uninfected L-cells. Each symbol represents the mean value of six animals with one standard deviation.

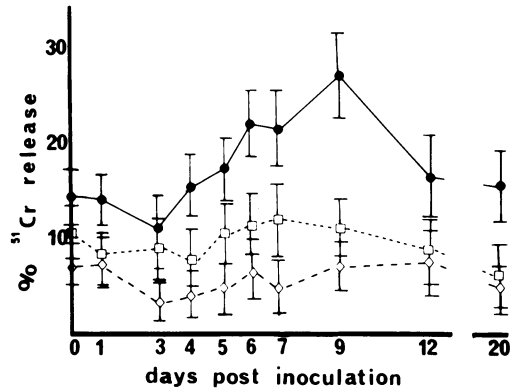


FIG. 7. Percentage of  $^{51}Cr$  release by spleen cells from animals infected intranasally on day 0 with the A/WSN ( $H_0N_1$ ) strain of influenza virus against the following target cells: (●) A/WSN ( $H_0N_1$ )-infected L-cells; (□) A/Jap ( $H_2N_2$ )-infected L-cells; (◇) uninfected L-cells. Each symbol represents the mean value of six animals with one standard deviation.

heterologous virus strain A/Jap ( $H_2N_2$ ) (Fig. 6 and 7). To further investigate this specificity, the recombinant viruses A/Jap-NWS ( $H_2N_1$ ) and A/NWS-Jap ( $H_0N_2$ ) were used to infect L-cells. No significant cytotoxicity was observed against the recombinant virus-infected target cells bearing the homologous neuraminidase,  $N_1$  (Fig. 8), to the immunizing virus. However, the A/WSN ( $H_0N_1$ )-immune lymph node cells showed a marked cytotoxicity against L-cells infected with the recombinant virus A/NWS-Jap ( $H_0N_2$ ), which contained the same hemagglutinin antigen as the immunizing virus.

## DISCUSSION

It is well known that protection against a subsequent exposure to influenza virus is greater after natural infection than when produced by subcutaneous vaccination, even when the serum antibody level is of equal magnitude. This suggests that some local factor in the respiratory tract may be involved (5, 21). Many studies have shown that specific antibodies present in secretions of the respiratory tract could be correlated with protection against reinfection by the homologous virus (17), but the relationship between secretory antibody levels and protection could be indirect, since some workers have reported protection in the absence of secretory antibody (1; P. A. Small, R. H. Waldman, J. C. Bruno, and G. E. Gifford, *Fed. Proc.* 32:104, 1973). To explain the anomalies between the humoral antibody response and resistance to influenza infection, CMI has been implicated as a possible protective mechanism.

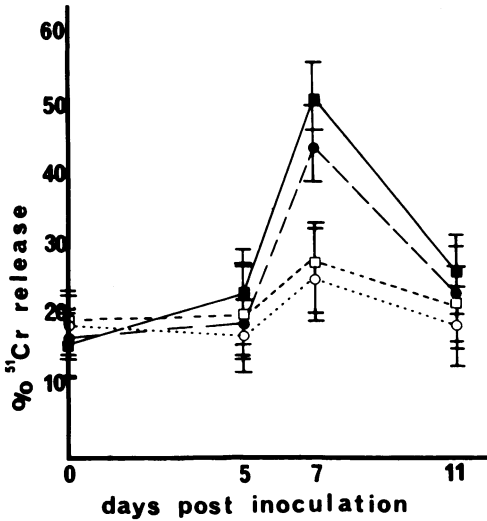


FIG. 8. Percentage of  $^{51}\text{Cr}$  release by lymph node cells from animals infected intranasally on day 0 with the A/WSN ( $\text{H}_0\text{N}_1$ ) strain of influenza virus against the following target cells: (■) A/WSN ( $\text{H}_0\text{N}_1$ )-infected L-cells; (●) A/NWS-Jap ( $\text{H}_0\text{N}_2$ )-infected L-cells; (□) A/Jap-NWS ( $\text{H}_2\text{N}_1$ )-infected L-cells; (○) A/Jap ( $\text{H}_2\text{N}_2$ )-infected L-cells. Each symbol represents the mean value of six animals with one standard deviation.

CMI is mediated by the activity of thymus-derived lymphocytes and is expressed by the elaboration of lymphokines that influence effector cell populations or by T-cell-mediated lysis of virus-infected cells (27).

The results of this study showed that *in vitro* techniques may be used to measure lymphocyte-mediated cytotoxicity to an acute viral infection, influenza.

The release of  $^{51}\text{Cr}$  from target cells was used as an index of cell damage by lymphocytes, taking into account nonspecific release of radioactive chromium. The advantage of the isotope release method was that gross cell damage could be detected as well as changes in permeability of the cell membrane resulting from interactions with effector cells (18). Significant cytotoxicity was observed against influenza-infected L-cells by lymphocytes from animals infected with the homologous virus; no significant reactivity was shown by lymphocytes from uninfected mice. Cytotoxicity was not evident against heterologous virus-infected target cells (Fig. 6 and 7), nor was it evident after treatment of target cells with complement and serum collected from these animals.

Cells from the cervical and mediastinal lymph nodes of animals inoculated with the strain of influenza virus, antigenic type  $\text{H}_0\text{N}_1$ ,

exhibited cytotoxicity against the homologous virus-infected target cells on day 5 post-inoculation, reached a peak at day 7, and rapidly declined to normal levels by day 20. The titer of infectious virus in the lungs commenced to decline after day 5 (Fig. 1), the time when cytotoxic lymphocytes began appearing in the lymph nodes draining the lungs (Fig. 6). This suggested a relationship between the presence of sensitized lymphocytes and control of viral multiplication in infected tissues. However, the *in vivo* significance of this phenomenon in influenza infection is uncertain since there is no evidence to indicate that animals or humans deficient in this type of immunity (CMI) are more susceptible to acute respiratory disease.

Sensitized T-cells appeared in the spleen on day 9 postinfection, shortly after maximum cytotoxicity was detected in the pulmonary lymph nodes of infected animals. The spleen response could have been due to cytotoxic lymphocytes escaping from the pulmonary areas of infection or to stimulation of spleen cells by virus particles carried there by the circulatory system. Cytotoxicity of both lymph node and spleen cells declined rapidly after day 9. Whether the response decreased because there were insufficient virus-infected cells in the convalescent animal to maintain the response or whether there were other regulatory factors has not been investigated.

Lymphocytes from the lymph nodes of A/WSN ( $\text{H}_0\text{N}_1$ )-inoculated animals were cytotoxic for the recombinant-infected L-cells bearing the homologous hemagglutinin (A/NWS-Jap ( $\text{H}_0\text{N}_2$ )) to the infecting virus (Fig. 8). There was no significant response against target cells bearing the homologous neuraminidase antigen [A/Jap-NWS ( $\text{H}_2\text{N}_1$ )].

Leukocyte migration inhibition was found to be maximal in pulmonary lymph nodes on day 8 and in the spleen by day 13 after influenza inoculation (Fig. 2 and 3). Thus, as in other studies (12, 23), local application of antigen led to an earlier appearance of local CMI.

A local secondary response was shown by pulmonary lymph node cells in the leukocyte migration inhibition test (Fig. 2); however, no secondary response was observed in the lymphocyte-mediated cytotoxicity assay.

Migration enhancement was shown against homologous viral antigens by lymph node cells on day 3 post-inoculation and in the spleen until day 6 (Fig. 2-5). It has been suggested that increased migration, when compared with appropriate controls, might be a measure of antigen stimulation of the sensitized lymphocytes. Svejcar et al. (22) have proposed that the phenomenon of enhancement of migration was

due to the presence of minute quantities of leukocyte migration inhibition factor, which at low concentrations may cause this effect. However, a factor that seemed to be responsible for the enhanced migration of leukocytes in the presence of specific antigen has been isolated by polyacrylamide gel electrophoresis of supernatants from antigen-stimulated lymphocyte cultures (26). This nondialyzable, heat-stable molecule was present in the  $\beta$ -globulin fraction of the supernatant and was called the migration enhancement factor. Their study showed that migration enhancement factor was produced in response to specific antigen (in this case, purified protein derivative) as early as 1 h after exposure to the sensitizing antigen by the same cell population that produced leukocyte migration inhibition factor.

The leukocyte migration inhibition response of animals inoculated intranasally with influenza strain A/WSN ( $H_0N_1$ ) was specific for the homologous hemagglutinin antigen, and little activity was observed against the recombinant virus containing the homologous neuraminidase with a heterologous hemagglutinin, A/Jap-NWS ( $H_2N_1$ ) (Fig. 4 and 5).

Thus, specificity against the homologous hemagglutinin antigen to the infecting virus was observed in both tests used as correlates of CMI in these experiments. It is known that the neuraminidase antigen can be detected on the surface of influenza-infected cells at approximately the same time postinfection as the hemagglutinin (15). However, the relative proportion of hemagglutinin and neuraminidase on the surface of the virion is known to vary between different virus strains. The neuraminidase antigen elicits humoral antibody either when inoculated as the isolated component or as a part of the complete virion (13). It is well known that there are qualitative differences in the induction process for antibody production and CMI (16). Therefore, more comprehensive experiments are required before any conclusions can be reached to explain this phenomenon.

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