Cytotoxic T-Cell and Antibody Responses to Influenza Infection of Mice

(Accepted 9 January 1979)

SUMMARY

The immune response to influenza infection was evaluated in mice using recently developed techniques to measure the induction of cytotoxic thymus-derived (T) lymphocytes and complement-dependent cytolytic antibody, locally and systematically, during primary and secondary immunization. Cytolytic antibody responses were compared to antibody titres measured by haemagglutination-inhibition (HI) and neutralization in the same samples. The development of these responses was also correlated with the titres of virus in the lung, in an attempt to further define the role of these host immune responses which can kill virus infected cells during recovery from influenza infection in vivo.

The cytolytic immune response to influenza infection may play an important role in eliminating acute infection of the host by lysing infected cells. Protective immunity against influenza infection, as described by Schultz (1975), has been shown to be due to the development of antibody to the major surface influenza antigen, the haemagglutinin. In previous studies (Ennis et al. 1977a, b, c), we have reported that influenza infection or vaccination generates a cytotoxic T-cell response which is specific to the haemagglutinin antigen subtype expressed on the infected target cell. Others (Effros et al. 1977; Doherty et al. 1977; Zweerink et al. 1977) have presented evidence which demonstrated a cross-reactive cytotoxic T lymphocyte response to influenza by using transformed target cells which were not permissively infected by influenza viruses. We use murine kidney cells which are permissively infected as the target cells in the cytotoxic T-cell assay. We have also demonstrated (Verbonitz et al. 1978) a haemagglutinin-specific, complement-dependent, cytolytic antibody response to influenza infection. The current studies were designed to examine these immunological responses to influenza infection, as well as other more well-known host responses.

The recombinant virus MRC-9 (H3N2) was used in all experiments. It was derived from A/Port Chalmers/1/73 (H3N2) and A/PR/8/34 (H0N1), and was provided by Dr Geoffrey Schild, Holly Hill, England. This was adapted for growth in mice by repeated intranasal passage of infected lung tissue. Stocks of virus for use in immunization and infection of target cells were prepared in fertile hens' eggs. Three-week-old BALB/c mice were inoculated intranasally (i.n.) with 5 LD$_{50}$ of this virus, diluted in PBS containing 0.1% bovine serum albumin. Control mice received diluent i.n. Fifty days after the primary inoculation, some of the previously infected and control mice were given either an i.n. challenge dose with 10 LD$_{50}$ of the same virus or an intraperitoneal (i.p.) booster of 5000 haemagglutinating units of an A/Port Chalmers formalin-inactivated whole virus vaccine. Lungs were homogenized and assayed for the presence of virus in the allantoic fluid of fertile hens' eggs.

Spleens were removed from infected and control mice at various times after virus inoculation and prepared as previously described (Ennis, 1973). Lymph nodes were removed from the anterior cervical region of control and infected mice and prepared as described previously (Ennis et al. 1977a). These lymphocytes were tested at a 100:1 ratio on MRC-9...
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(H3N2) infected syngeneic kidney cells in an 18 h $^{51}$Cr release cytotoxicity assay as previously described (Ennis et al. 1977a).

Mice were anaesthetized with ether, the trachea surgically exposed, and a small incision made in the upper section to obtain bronchial secretions. A short piece of polyethylene tubing (Clay Adams I. D. 0.86 mm) was inserted into the trachea and the trachea and bronchi were washed several times with 0.5 ml of warm Hanks' balanced salt solution (HBSS) using a 1.0 ml syringe and 20 gauge needle. Samples contaminated with blood were discarded. The secretions were then sonicated at 80 W/s for 1 min and spun down at 2000 rev/min for 15 min to remove cellular debris. The supernatant was concentrated tenfold with an Amicon concentrator (Amicon Corp., Lexington, Mass.) and assayed for the presence of antibodies.

Sera and bronchial secretions were assayed for haemagglutination-inhibiting (HI), neutralizing and complement-dependent cytolytic antibody as previously described (Verbonitz et al. 1978). The cytolytic antibody test, like the lymphocyte test, was performed using infected BALB/c kidney target cells, in a 2 h $^{51}$Cr release cytotoxicity assay. Blood-free 10 x concentrated bronchial secretions were assayed on immunodiffusion plates (Meloy Laboratories Inc., Springfield, Va.) for mouse IgG, IgA or IgM. At 24 h the diam. of the precipitin rings were measured and mg% values were calculated using reference murine immunoglobulins from Meloy Laboratories as a standard. Total protein in the bronchial secretion was determined according to the procedure of Lowry et al. (1951).

Following primary infection (Fig. 1), the highest mean virus titres in the lung (6.5 log$_{10}$ per 0.1 ml) was seen on day 4 and decreased to 5.5 log$_{10}$ by day 6. No virus was isolated after this, following primary infection, and none was detected following subsequent live virus challenge inoculation with 10 LD$_{50}$ on day 50.

The humoral cytolytic and HI antibody responses to primary infection with virus were first detected in this experiment on day 8 (Fig. 1). Neutralizing antibody was detected on day 12 and then rose to generally higher titres than the HI and cytolytic antibodies. These serum antibodies reached a peak titre on day 30 and remained fairly stable through the next month. Boosting mice with vaccine i.p. produced a much better secondary reaction than did the administration of live virus i.n. On day 64, 14 days after the secondary inoculation of vaccine, we saw a significant increase in antibody measured in all three assays (Neutralizing, 128 to 4096; HI, 64 to 427; cytolytic, 35 to 306). The challenge dose of live virus resulted in a significant antibody titre increase on day 64, only when measured by plaque neutralization (128 to 1024).

Cytotoxic lymphocytes (Fig. 1) were detected in the spleen as early as day 4 after infection in this experiment [12-0 % specific immune lysis (SIL), $P < 0.05$ by Student's $t$ test] in comparison with lymphocytes from uninfected animals. The primary T-cell response peaked on day 8 (24.4 % SIL, $P < 0.001$) and was again statistically significant on day 12 (20.5 % SIL, $P < 0.05$), decreasing to insignificant values by day 14. Significant secondary T-cell responses were seen following both the i.n. administration of live virus and i.p. inoculation with inactivated virus on all 3 days of sampling following the booster dose.

We were first able to detect antibody in concentrated bronchial secretions (Fig. 2) on day 12 as compared to their detection in sera by day 8. The local antibody response also peaked on day 14 or 30, depending on the assay, but differed from the primary humoral antibody response by continuing to decline to undetectable levels by the end of the test period. Similar to the humoral antibody response, a better secondary local antibody response was observed with the i.p. vaccine inoculation. Secondary complement-dependent cytolytic antibody responses could not be measured due to a lack of samples. Antibody titres measured in the bronchial secretions were lower than those found in sera, probably
Neutralizing antibody

HI antibody

Complement-dependent cytolytic antibody

Cytotoxic spleen cells

Virus titre in lungs

< 8

< 8

< 8

< 8

< 0.5

0 4 6 8 12 14 30 50 53 57 64

Time after infection (days)

< 8

< 8

< 8

< 8

< 0.5

0 4 6 8 12 14 30 50 53 57 64

Time after infection (days)

Neutralizing antibody

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Time after infection (days)

Fig. 1. Systemic immune responses to influenza infection as measured in the serum and the spleen. Reciprocal antibody titres represent the mean of three or four individual assays performed on serum pools obtained from ten mice sacrificed on the day indicated after infection. The percentage specific immune lysis (% SIL) was obtained from the mean lysis of eight replicate wells of lymphocytes pooled from 10 spleens. ●●●, Primary live virus inoculation given i.n.; △△△, live virus challenge inoculation given i.n.; □□□□□, vaccine booster inoculation given i.p.

Fig. 2. Local immune responses to influenza infection measured in the bronchial secretions (antibody) and cervical lymph nodes (cytotoxic cells). Reciprocal antibody titres represent the mean of three or four individual assays performed on concentrated bronchial secretions pooled from ten mice sacrificed on the indicated day after infection. The %SIL for cytotoxic lymphocytes was obtained as in Fig. 1. ●●●, Primary live virus inoculation, i.n.; △△△, live virus challenge inoculation, i.n.; □□□□□, vaccine booster inoculation, i.p.

due to the dilution involved in collecting the secretions despite subsequent tenfold concentration of samples.

A significant cytotoxic lymphocyte reaction in the cervical lymph node cells was only detected on day 4 after primary infection (Fig. 2). In previous experiments we have detected cytotoxic T-cells in cervical lymph nodes from 3 to 6 days after infection (Ennis et al. 1977a).
A comparable degree of specific immune lysis was measured using these local lymph node cells 3 and 14 days after both types of booster inoculations.

Bronchial secretions were also tested for IgG, IgA and IgM by immunodiffusion. Only IgG values could be measured and these values were rather low. A peak value of 17 mg% was reached on day 8 and steadily declined to undetectable values by day 53. Both booster types of inoculations stimulated a temporary increase of immunoglobulin levels. Surprisingly the vaccine booster gave higher values than did the live virus challenge. Lowry protein assay determinations of the same samples showed similar increasing (peak on day 8 of 2200 μg/ml) and decreasing values paralleling those of IgG at similar times after primary and secondary inoculation.

Comparison of the local and the systemic immune response revealed an early cytotoxic lymphocyte response in local draining lymph nodes which fell rapidly, but persisted until day 14 in the spleen. A comparison of the mean antibody titres, including cytolytic, HI and neutralizing antibodies showed that the primary serum antibody responses were detected early (days 6 to 8) and remained stable throughout the observation period, whereas the primary local antibody response was not as long-lived. A quantitative comparison of the systemic and local antibody titres, however, would not be valid due to the difficulty of measuring the dilution involved in obtaining the bronchial secretions. Both local and systemic T-cell responses were detected earlier than the antibody responses at a time when infectious virus was still present in the lung. This might indicate that cytotoxic lymphocytes have an important role in clearing virus from the lung during primary influenza infection.

The booster inoculation of inactivated vaccine had a much higher antigen content (approx. 10^{12} virus particles) than the live virus challenge (approx. 10^4 EID_{50}) and did produce increased antibody titres systemically and also in the local bronchial secretions. Protection against influenza virus infection has been shown in mice and ferrets (Fazekas de St Groth & Donnelly, 1950; Shore et al. 1972) to be more closely related to antibody in bronchial secretions than to serum antibody. We detected IgG in bronchial secretion but failed to detect IgA, as have others, in ferrets (Potter et al. 1972). Scott & Sydiskus (1976), however, did detect IgA and IgM in murine bronchial secretions after an aerosol of small particles of influenza virus was used. Cytotoxic T-cells in the local lymph nodes and spleen were, however, slightly higher in their response to live virus challenge than after vaccine boosting.

Our purpose in comparing these local versus the systemic responses was to determine where and when influenza virus antigen stimulated cytotoxic T-cell and cytolytic antibody responses during the course of infection and recovery. These host responses, both antibody-mediated and T-cell-mediated, have been shown in vitro to effectively eliminate the infected cells. The present experiments as well as previous studies with influenza infection (Ennis et al. 1977c, 1978; Verbonitz et al. 1978) indicate that local and circulating antibodies and local and systemic cytotoxic T-cells have receptors for recognizing the specific virus antigen expressed on the membrane of infected cells which they can lyse. In addition, the antigen-antibody complex with complement, as well as the sensitized T-cells, may act indirectly by elaborating chemotactic factors (Ward et al. 1972) which would result in the migration of phagocytes into the infected area to contribute to the elimination of the infection. These cytotoxic reactions can be immunopathological as well as beneficial to the host (Brier et al. 1971). In addition the development of antibodies to influenza has been shown to be T-cell dependent (Burns et al. 1975).

Recent studies have indicated a marked increase in the number of lymphocytes in the mouse lung at day 7 after infection, the time when peak lymphocyte cytotoxicity was detected and decreasing amounts of virus were isolated from the lung (Ennis et al. 1978; Yap & Ada, 1978). Other recent data from our laboratory, using complement-deficient mice,
have demonstrated that an intact complement pathway was important in the final elimination of infectious virus in vivo, possibly indicating the importance of complement-dependent cytolytic antibody in recovery from infection (Hicks et al. 1978).

Experiments with nude, T-cell deficient mice (Sullivan et al. 1976) or anti-lymphocyte-serum treated mice (Suzuki et al. 1974) have revealed that virus persisted in the lungs of infected immunodeficient mice for a longer period of time and was associated with delayed or decreased mortality and with a decreased production of antibodies. These reports imply that T-cells may have a detrimental as well as beneficial role in the immune response. These reports and the evidence of the need for complement and antibody to clear virus (Hicks et al. 1978) indicate that the immune response to influenza infection consists of an interplay of both humoral and cellular components, both of which appear important in the clearing of virus from the lung and in addition may contribute to pulmonary pathology.

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REFERENCES


*(Received 11 August 1978)*