Cell-Mediated Immune Response of Human Lymphocytes to Influenza A/USSR (H1N1) Virus Infection

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Cell-mediated immunity to influenza A/USSR (H1N1) virus was assessed by measuring transformation response and interferon production by Ficoll–Hypaque-purified peripheral blood lymphocytes from children and adults. Lymphocyte transformation was found to be related to the individual’s previous experience with H1N1 influenza virus. Lymphocyte cultures, obtained from adults who had their last contact with the H1N1 virus over 20 years ago, were able to transform when incubated with H1N1 influenza antigens. This response suggests that influenza virus can induce long-term cell-mediated immunity for periods of at least 20 years. Interferon produced by stimulated lymphocytes was found to be unrelated to previous contact with influenza H1N1 virus; seronegative as well as seropositive individuals were capable of producing interferon in response to viral antigens. The interferon produced was type I.

Lymphocyte transformation (LTF) and interferon production have been used to assess cell-mediated immune response to viral infections in humans (7, 8, 11, 14, 22, 23). However, the role of cell-mediated immunity in recovery and protection from viral infection is poorly understood. Blastogenic responses due to influenza have been demonstrated for up to 9 to 11 months after experience with the virus, but the duration of “memory” for transformation potential is unknown (2). In other viral systems, LTF memory is long lasting, 3 to 20 years for vaccinia (7) and over 40 years for mumps (21).

The present study examines the response of human lymphocytes to influenza A/USSR/77 (H1N1) strain. H1N1 virus last circulated in the United States in 1957 before reappearing with limited circulation in January 1978 (9). Therefore, antibodies to H1N1 antigens currently found in the sera of most adults reflect contact with virus more than 20 years ago. Lymphocytes of seropositive adults, seronegative children, and children undergoing primary infection with influenza A/USSR virus were examined for transformation and interferon production. Samples for study were obtained during the summer and fall of 1978 after a limited initial A/USSR (H1N1) outbreak, confined almost exclusively to those less than 25 years of age.

MATERIALS AND METHODS

Patient population. Adults were healthy Vanderbilt hospital employees, ages 27 to 49 years, with no history of influenza infection or vaccination since January 1957. Children undergoing natural infection were 1 to 6 years old and enrolled in the Vaccine Clinic at Vanderbilt Hospital.

Preparation of lymphocyte cultures. A 5-ml specimen of venous blood was drawn and mixed in a heparinized syringe (10 U/ml). The blood was layered over a Ficoll-Hypaque gradient and spun at 600 × g for 40 min (1). The interface containing the lymphocyte fraction was removed and the cells were washed and cultures seeded with 10^6 cells per ml in McCoy 5A medium supplemented with 10% heat-inactivated gamma calf serum. Antigen or mitogen was added, and the cultures were incubated at 37°C in a 5% CO2 incubator at 95% humidity.

Influenza virus antigens. The influenza virus used in the study was isolated in this laboratory in a continuous canine kidney cell line (MDCK) (16) during the 1978 winter epidemic. The virus was inhibited by antiserum to A/USSR/77. Other influenza viruses isolated in Vanderbilt Hospital during that time were fully characterized by the Center for Disease Control, Atlanta, Ga., and resembled the prototype A/USSR/90/77 virus.

The virus was passaged three times in MDCK cells before use. Culture fluid from infected cells showing complete cytopathic effect was clarified by low-speed centrifugation (1,000 × g for 15 min), and virus recovered from the resulting supernatant was inactivated by ultraviolet germicidal light at a distance of 15 cm for 4 min. This treatment completely inactivated 10^8 50% tissue culture infective doses of influenza A/USSR virus. Quantitation of viral antigens was performed by hemagglutination with chicken erythrocytes. One hemagglutination unit in this system equals 10^2 50% tissue culture infective dose. Uninfected supernatants from MDCK cell cultures were handled identically and served as controls. Twenty-five hemagglutination units of influenza A/USSR virus or an equivalent control volume was added to 10^6 lymphocytes. Twenty-five hemagglutination units was found to be an optimal concentration for lymphocyte transformation in preliminary experiments.

PHA. A 2-μg amount of phytohemagglutinin-P
LTF assay. The blastogenic response of stimulated lymphocytes was assayed by measuring the incorporation of 2.5 μCi of [3H]thymidine (6.7 Ci/mmol, New England Nuclear Corp., Boston, Mass.) into trichloroacetic acid-precipitable material.PHA-stimulated cells were harvested at 72 to 96 h after stimulation, whereas viral antigen-stimulated cells were harvested at various intervals as described below. Duplicate cultures of 5 × 10⁶ cells were labeled for the last 24 h in culture and harvested by centrifugation (1,000 × g for 10 min). Supernatants were collected and frozen at −20°C for interferon assay. Cell pellets were precipitated with 10% trichloroacetic acid and filtered onto Whatman GF/A filter paper. Filters were washed three times with cold 5% trichloroacetic acid and once with cold 95% ethanol, dried at 37°C, and put into 5 ml of toluene-base scintillation fluid. Samples were assayed in a Packard Tri-Carb liquid scintillation counter. Mean incorporation was determined for each group, and a stimulation ratio was derived by dividing the mean counts per minute incorporated in the presence of the viral antigens (or PHA) by the mean counts per minute incorporated in the control cultures.

Interferon assay. Supernatants from lymphocyte cultures were initially screened for interferon activity by using the microculture assay described by Haar et al. (11). Final interferon titers were obtained by using a plaque reduction test employing human foreskin fibroblasts challenged with vesicular stomatitis virus (17). Titers were expressed as reciprocals of the highest dilution that inhibited 50% of vesicular stomatitis virus plaque-forming units. A standard reference of human interferon (NIH G-023-901-527) was included in each assay. One unit of interferon in our assay was the equivalent of 2 to 3 U of the reference standard. The lowest dilution of each sample tested was 1:10, establishing the lower limit of sensitivity of our test as 10 U/ml.

Interferon samples were characterized as follows. Samples were dialyzed against 500 volumes of glycine buffer (pH 2) at 4°C for 24 h, followed by dialysis at pH 7.2 for further 24 h. Controls were dialyzed at pH 7.2 for 48 h. Heat lability was tested by incubating the samples at 56°C for 60 min. Samples were centrifuged at 100,000 × g for 90 min to determine the interferon activity in supernatants. Species specificity was tested by the plaque reduction technique with vesicular stomatitis virus and mouse L cells.

Antibody determinations. Hemagglutination inhibition (HAI) antibody titers were performed in the standard manner (4) by using the microtiter system. Individuals were considered seronegative when their HAI titer was 1:8 or less.

RESULTS

Kinetics of LTF and interferon production. The in vitro kinetics of LTF and interferon production were determined in children and adults having A/USSR HAI antibody titers ranging from 1:16 to 1:128. Lymphocytes were assayed on days 1 to 7 after antigenic stimulation with 25 hemagglutination units of inactivated influenza A/USSR virus. A representative kinetic curve of an adult volunteer with HAI serum titer of 1:128 is shown in Fig. 1. Maximum LTF occurred 3 days after antigen stimulation and declined thereafter. Cultures stimulated with PHA demonstrated peak values of transformation between 72 and 96 h after stimulation. Interferon production by the same cultures was initially detected on day 1 and reached a peak on day 5 after stimulation.

Lymphocytes incubated with control antigen showed a low uptake of [3H]thymidine, and interferon production was below detectable levels (10 U/ml). The findings of maximal LTF and interferon responses defined the optimal experimental conditions in this system and were selected for further studies.

LTF of seronegative children, seropositive children, and seropositive adults to influenza H1N1 virus. LTF response to influenza A/USSR virus was measured in 18 seronegative children, 12 seropositive children, and
12 seropositive adults. All seropositive children had had their first experience with the A/USSR virus 2 to 12 months before testing. The adult group was individuals outside of the susceptible age range without clinical evidence of exposure to A/USSR in early 1978. In 7 of 12 seropositive adults, sera frozen 1 to 3 years before January 1978 were available. HAI antibody titers to H1N1 influenza virus showed no significant, ≥fourfold, changes in titer in any of the individuals when their sera collected before and after the A/USSR outbreak of winter 1978 were compared.

Stimulation ratio values measured in the three groups of patients are illustrated in Fig. 2. No significant LTF response was observed in the seronegative group with A/USSR antigen. Intrinsic transformation capability was present, however, as determined by studies performed with PHA. Seropositive children showed significant increases in the LTF response when compared with seronegative children (P < 0.05, Duncan's multiple range test). The response of se-

Relation between HAI antibody level and lymphocyte transformation. Serum HAI antibody to influenza A/USSR virus was determined in 12 adults and 30 children and correlated with LTF response to A/USSR viral antigens (Fig. 3). A significant direct quadratic correlation was observed between the LTF response and serum HAI antibody titer (r = 0.88, P < 0.05, Duncan's multiple range test). HAI titers from seropositive children were, in general, lower than titers found in adults.

Relation between HAI antibody levels and interferon production. Interferon production by children's and adult's lymphocytes was measured as a response to in vitro incubation with influenza A/USSR-inactivated virus (Fig. 4). All lymphocyte cultures were capable of producing interferon. No correlation was found between serum HAI titer and interferon production (r = 0.1). The interferon produced by the lymphocytes of both seropositive and seronegative individuals was resistant to pH 2 and heat inactivation at 56°C for 60 min. It was not sedimented at 100,000 × g for 90 min and showed species specificity by lack of inhibition of vesicular stomatitis virus plaque formation on mouse L cells. These tests indicate that the interferon produced was type I and unrelated to immune status.

**DISCUSSION**

In January 1978 the H1N1 strain of influenza virus (A/USSR) was isolated in the United States for the first time in 20 years (9). H1N1 strains circulated from 1947 to 1957 suggesting that the population over 20 years of age would be immune to these antigens from past exposure. However, children were expected to lack antibodies to this virus. Subsequent epidemiological observations revealed that illness was primarily confined to individuals younger than 25 years of
adults appeared to reflect long-lived memory of sensitized lymphocytes to influenza H1N1 virus. The age of the adults, lack of influenza illness, and stable serological titers (in the 7/12 testable) suggest the recent reexposure to A/USSR before lymphocyte testing was unlikely. More likely is that the initial H1N1 sensitization was enhanced by subsequent infection with other unrelated influenza strains; however, the blastogenic response to A/USSR virus was directly correlated with the present H1N1 HAI antibody titer. These results are in accordance with the observations of Cate and Kelly (2) who found a direct correlation between HAI antibody titer to influenza A/Hong Kong/68 and LTF. However, these results disagree with those of Cole and Molyneux (3), who were unable to find any correlation between LTF to influenza B/Mass/3/66 and circulating HAI antibodies. This discrepancy may reflect differences in HAI or lymphocyte blastogenic response to type A and B influenza viruses.

The LTF response demonstrated in this study is characterized by relatively low levels of blastogenesis even in individuals with high serum HAI titers. Similar low reactions were observed with other influenza types such as A/Hong Kong/68 (H3N2) (2), A/England/61 (H2N2), B/England/69 (5), and B/Mass/3/66 (3). These low levels may reflect the short life of maximal LTF response after influenza infection observed by Dolin et al. (6). The early detection of the LTF response even before HAI antibody rise (6) and the long-lived memory as shown in this study make this response a potentially important factor in host’s defense mechanism against viral infections.

The interferon produced by Ficoll–Hypaque-purified lymphocytes was stable to pH 2 treatment and to heat inactivation and was classified therefore as type I. Interferon production was not related to immune status of children and adults studied in this trial or in trials with Varicella-Zoster and herpes simplex viruses when Ficoll-Hypaque lymphocytes were used (11, 14). The macrophage-lymphocyte technique useful for detection of immune interferon (19) was unsuitable for these studies in small children due to limited availability of blood.

Type I interferon, as determined by heat and acid stability, may play an important role in recovery from influenza infection. Support for this is derived from the present study and from previous observations: (i) type I interferon is detected in sera and nasopharyngeal washings of patients after influenza infection (12, 13, 15, 18); (ii) no previous contact with the virus is required for lymphocytes to produce interferon; and (iii) interferon is detected in maximum

age during the initial circulation of the virus in the winter of 1978 (10).

This unique situation enabled us to study the effect on the cell-mediated immune system of influenza infection occurring more than 20 years previously. The LTF found in H1N1 seropositive
quantities as early as 5 days after induction by influenza virus.

We have shown that cell-mediated immunity to natural influenza infection is long lived and correlated with humoral immunity. Studies of the effect of vaccination with live, attenuated influenza vaccines on the cell-mediated immune system are reported in a companion paper.

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LITERATURE CITED