Lymphocyte Responses to Hemagglutinin and Neuraminidase Subunits of Influenza Virus

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The responses of peripheral lymphocytes to purified hemagglutinin and neuraminidase subunits and other components of an influenza A virus were measured in 21 normal adults and compared with antibody titers. All had influenza antibodies and demonstrated influenza antigen recognition by lympho-proliferative responses. There was a significantly greater response to the two purified influenza virus surface antigens, hemagglutinin and neuraminidase, than to either whole intact virus or its separated subviral core. No correlation between magnitudes of antibody titers and lymphocyte responsiveness was observed.

Cell-mediated immunity to influenza viruses has been demonstrated in humans by using the lymphocyte transformation (LT) assay (4, 5, 8), with either whole influenza virions or split-virus antigens as antigenic stimulant. The recently described method of selective solubilization of surface components from influenza virus (2, 3) permits the assessment of the comparative activities of certain components of the virion in the LT assay. Our studies assessed the LT responses to the mixture of the two influenza virus surface antigens, hemagglutinin (HA) and neuraminidase (NA), compared with responses to the core components. These data show that all of the antigen preparations stimulated lymphocytes from sensitized persons.

MATERIALS AND METHODS

Lymphocytes from 21 healthy adult volunteers, ranging from 19 to 39 years of age, were tested during a season in which no influenza was noted in the community (fall 1976); none had been immunized with influenza vaccine for at least 1 year. Venous blood samples were collected in heparin, and the lymphocytes were separated (10). As a source for unsensitized lymphocytes, 11 cord blood samples, processed as above, served as controls.

Egg-grown zonal purified influenza A/Hong Kong/68 (H3N2) as recombinant X-31 was used for preparation of viral antigen. By using the principle of selective solubilization (2, 3) and the cationic detergent cetyltrimethylammonium bromide, the following preparations were obtained: (i) HA + NA with HA activity of 2^{15} ; (ii) subviral particles (core antigens containing all internal viral proteins) with residual HA activity of 2^1 . A third antigen preparation consisted of untreated and concentrated whole virus with HA activity of 2^{14} . Polyacrylamide electrophoresis was used to assess the biochemical purity of the preparations. Stock solutions of the above antigen preparations were

assayed for protein content with Folin reagent and showed HA + NA at 0.15 mg/ml, core at 0.30 mg/ml, and whole virus at 0.42 mg/ml.

LT studies were modified from those previously described (10). After bringing cetyltrimethylammonium bromide-prepared antigens to equivalent concentrations (all antigen preparations began with the same amount of whole virus and were brought to equivalent dilutions), all antigens were then used in dilutions of 10^{-1} , 10^{-2} , and 10^{-3} . Each lymphocyte culture tube had a 5-ml total volume and contained 3×10^6 lymphocytes in RPMI 1640 medium (Grand Island Biological Co.), 20% fetal calf serum, 0.16 mg of gentamicin, and 0.1 ml of antigen or phosphate-buffered saline as a control. Antigen-containing tubes were tested in duplicate, and control tubes were tested in quadruplicate. All cultures were incubated in 5% CO₂ for 5 days, the optimal time for maximum responses; at 4 h before harvest, 1 μ Ci of [³H]thymidine was added. The cells were harvested onto filter paper and washed, and acid-insoluble material was precipitated with 5% trichloroacetic acid. The radioactivity was counted in a liquid scintillation counter (Packard). In addition, phytohemagglutinin (PHA) responses were evaluated to ensure normal lymphocyte reactivity. These cultures were incubated for 3 days, the optimal time, before harvest. Results of all assays were expressed as the ratio between stimulated and control cultures. Results were compared by using the Student *t* test for paired or nonpaired observations.

All sera were tested for HA-inhibiting (HAI), NA-inhibiting (NAI), and complement-fixing (CF) antibodies to the A/Hong Kong/68 virus of influenza A soluble antigen (W.H.O. Reference Laboratory, Center for Disease Control) and in the HAI test to influenza A/Port Chalmers/73 (H3N2) as well (7).

RESULTS

The PHA stimulation ratios found in lymphocytes from 21 adults were 123 ± 61 (mean $\pm$ standard deviation) and ranged from 26 to 298.

Responses to the influenza antigens alone and in combination are shown in Fig. 1. Significantly higher stimulation ratios were obtained with surface antigens or the combined preparation of surface antigens plus core than were obtained by whole virus or core ($P < 0.005$). The responses with intact whole virus were similar to those with core preparations alone. Varying the antigen concentration did not appreciably change the differences in responsiveness of lymphocytes to the various antigen components.

Eleven cord blood specimens showed LT responses to PHA ranging from 2 to 41 (mean, 17 ± 15). The LT responses with influenza virus antigens are shown in Fig. 2. All responses were of low magnitude, with no difference among the antigens tested. The mean stimulation ratios of cord blood lymphocytes to influenza antigens were significantly lower than those obtained with adult lymphocytes ($P < 0.005$).

Table 1 summarizes the results of all serological studies on both adult and cord sera. All

adult sera had HAI and NAI antibody titers against the influenza Hong Kong/68 (H3N2) virus, indicating prior sensitization. All but one of these sera showed detectable ($>1:4$) but lower antibody titers against the more recent A/Port Chalmers/73 (H3N2) strain. All adult sera possessed CF antibodies to influenza A. The antibody titers to HA and NA in cord blood samples were uniformly lower than adult titers; CF antibody titers were of similar magnitude.

There was no correlation between the responsiveness of lymphocytes and the serum HAI antibody titer to either the A/Hong Kong/68 or the A/Port Chalmers/73 virus (Fig. 3).

DISCUSSION

Although sensitized lymphocytes usually respond to influenza antigens in the LT assay (5, 8), the particular antigens which elicit these responses have not been previously determined. Our studies showed that adult-derived lymphocytes respond to all of the antigen preparations used. The greatest responses were, however, to the surface antigen subunits, HA + NA, and to the antigen preparation containing these surface antigens plus the subviral core. The responses to

FIG. 1. Stimulation ratios from LT assay of adult-derived lymphocytes incubated with various influenza A/Hong Kong/68 antigens. SU, HA + NA; C, core; WV, whole virus.

FIG. 2. Stimulation ratios from LT assay of cord blood-derived lymphocytes incubated with various influenza A/Hong Kong/68 antigens. SU, HA + NA; C, core; WV, whole virus.

TABLE 1. Serological tests on 21 adult-derived and 11 cord blood-derived sera for antibodies against influenza

Antibody test	Antigen used	Geometric mean antibody titer ^a (range)	
		Adults	Cord bloods
HAI	A/Hong Kong/68	378 (16-2,056)	13 (<4-32)
HAI	A/Port Chalmers/73	23 (<4-128)	6 (<4-16)
NAI	A/Hong Kong/68	81 (10-130)	13 (<3.2-33)
CF	Influenza A soluble antigen	28 (8-64)	21 (8-32)

^a Reciprocal titer.

FIG. 3. Relationship of stimulation ratios in LT assays and HAI antibody titers with surface antigens of A/Hong Kong/68 influenza virus (correlation coefficient, 0.3601; $t = 1.68$; $P > 0.10$).

the whole virus were significantly lower. Furthermore, the core antigen preparation gave results similar to those obtained with whole virus.

One interpretation of our findings is that the surface antigen subunits are the major stimulants in the LT assay. However, an important alternative explanation would be that the detergent processing of the virus to produce surface antigen subunits has released a larger number of particles in the surface antigen preparation than in the whole virus or core preparations. Although we used the HA activity as a basis for making equivalent preparations, there definitely may be HA and NA monomers or dimers which may not cause erythrocyte agglutination (6) and, hence, be undetected in the HA assay. Furthermore, the intact virus with its spacially restricted HA and NA subunits may interact with primed lymphocytes as one unit or may require processing before it is stimulatory. Thus, we feel that we have shown that purified subunits of HA and NA are stimulatory for lymphocytes, but they may not necessarily be the major stimulants.

The finding that the core antigens were stimulatory and gave results similar to those with whole virus deserves comment. We assume that the core antigens are themselves stimulatory since our preparation was pure according to biochemical criteria. Alternatively, residual trace amounts of HA or NA antigen on the core

surface could account for the low stimulation with core.

In these studies, lymphocytes from adults previously sensitized to the influenza virus antigens were used. Serological studies confirmed sensitization in the adult donors; all adults had antibody titers to the A/Hong Kong/68 virus surface antigens and influenza A virus soluble antigen. Cord blood lymphocytes were chosen as nonsensitized control cells. Cord bloods also contained anti-influenza antibodies, but these were presumably maternally derived immunoglobulin G class antibodies which had crossed the placenta (9).

All cord blood samples showed PHA responses, but there was no significant response to any of the influenza antigens tested. Such results are consistent with the concept that these cord lymphocytes were not previously sensitized to influenza antigens. We have previously shown that during influenza outbreaks some fetal lymphocytes are presumably sensitized in utero. These lymphocytes showed positive responses to influenza antigens when tested later at birth (9).

In the present studies, there was no correlation between the magnitude of LT responses to influenza antigens and the titers of antibodies against different influenza virus components. Other have also failed to show a correlation between HAI antibody titer and lymphocyte reactivity using influenza B (5). These data suggest that measuring circulating antibodies against influenza and responsiveness of lymphocytes are unrelated assessments of sensitization.

We believe that our findings further emphasize the importance of immunity to the surface antigens of influenza. Antibodies against HA and NA are clearly of protective value but appear to be short-lived. Cell-mediated immunity to surface antigens in particular is now documented by our study, and it remains to be determined how long such immunity can persist. One approach is to measure LT responses to older types of influenza A virus and compare responses with those to more recent types. Elevated LT responses to older types could in part account for the lasting clinical protection against older strains noted in older persons. Studies to this end are now under way.

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