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Increased Influenza Pneumonia Mortality of Mice Adoptively Immunized with Node and Spleen Cells Sensitized by Inactivated but not Live Virus

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Syngeneic mice adoptively immunized intravenously with 25 million washed node and spleen cells from donors vaccinated subcutaneously with formolized influenza A PR8 had a higher mortality with influenza pneumonia after challenge with homologous virus than occurred in recipients of similar cells from unsensitized donors, and this increased mortality was prevented by treatment of the sensitized cells with antithymocyte serum. Mice adoptively immunized with cells from donors vaccinated with formolized influenza A PR8 also had a higher mortality than recipients of unsensitized cells after challenge with heterologous influenza B Lee. Mice who received PR8-sensitized cells and survived challenge with influenza B Lee developed antibody only to the challenge virus, and serum antibody titers to the challenge virus in surviving recipients of sensitized cells were similar to those of recipients of unsensitized cells in all studies. Influenza mortality of recipients of antibody-containing mouse serum after homologous virus challenge was similar to that of recipients of antibody-free mouse serum in this model. Washed node and spleen cells from donor mice who had survived respiratory infection or received subcutaneous vaccination with live influenza A PR8 and those from donor mice given typhoid vaccine subcutaneously all failed to alter mortality from that observed in recipients of unsensitized cells after challenge with influenza A PR8. These results suggest that subcutaneous vaccination with inactivated influenza establishes a reactivity of the cell-mediated immunologic system which can increase the severity of influenza infection of the respiratory tract under certain conditions, and that sensitization by live influenza fails to produce this effect.

Inefficient induction of secretory antibody is considered a major deficiency for parenteral immunoprophylaxis with inactivated respiratory virus vaccines (10). Moreover, respiratory disease of unusual severity has occasionally followed such vaccination. An example of the latter involved respiratory syncytial virus (RSV) in young children (7, 8), in whom failure of an inactivated vaccine to protect against natural infection was postulated to be due to lack of a secretory antibody response, and severe pneumonia was postulated to be due to toxic effects of the delayed reaction between viral antigens and antibody arriving from the circulation (2). A major rationale for the development of live attenuated respiratory virus vaccines has been to obtain higher secretory antibody titers and, presumably thereby, greater protection against illness.

The present report describes studies with influenza and adoptive immunizations in mice which suggest that the cell-mediated immunologic (CMI) system can also be important in determining how the host will respond to respiratory virus infection. The data, portions of which have been previously presented (Am. Rev. Resp. Dis., **107**:1085, 1973; T. R. Cate and N. G. Mold, Prog. Abstr. Intersci. Conf. Antimicrob. Agents Chemother., 14th, San Francisco, Calif., Abstr. 308, 1974), indicate that differences exist in how the CMI system responds on reexposure to influenza depending on whether the initial sensitization was with live or inactivated virus, and that, after sensitization by inactivated virus, thymus-dependent cells can participate in increasing mortality with influenza pneumonia in adoptive hosts. CMI effects such as these could potentially explain the severe RSV pneumonia in young recipients of inactivated vaccine and provide another rationale for the use of live virus vaccines.

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MATERIALS AND METHODS

Mice. Syngeneic, male CBA mice were purchased from Jackson Laboratories, Bar Harbor, Me., and were 8 to 10 weeks old when entered into the study.

Vaccines. Influenza A PR8 and influenza B Lee were purchased from the American Type Culture Collection, Rockville, Md. (VR95 and VR101, respectively). For inactivated vaccines, the viruses were grown in embryonated eggs, partially purified by absorption-wash-elution on chick erythrocytes, and inactivated with 0.05% formaldehyde at 37 C for 12 h. These inactivated vaccines usually had a hemagglutination titer of 1:256 or 1:512, although less potent vaccines gave similar results. For live vaccine, infected chorioallantoic fluid which had been stored at −70 C was diluted 1:25 in balanced salt solution; this dilution of live virus contains about 1,000 50% mouse lethal doses per 0.1 ml if inoculated intranasally, but causes neither deaths nor apparent disease after subcutaneous injection. Typhoid vaccine (U.S.P., Lilly) was purchased commercially and used as supplied.

Immunization. Mice were vaccinated subcutaneously with 0.1 ml of vaccine subcutaneously on both sides of the abdomen. Vaccinations were repeated at least three times at 2- to 3-week intervals with harvest of node and spleen cells 8 to 9 days after the last vaccination.

Node and spleen cells. Donor mice under ether anesthesia were exsanguinated via axillary vessels with collection of blood from axillary skin folds. Lymph nodes collected were cervical, axillary, inguinal, paratracheal, and mesenteric. Cells were freed from these nodes and the spleens into cold (4 C) medium by rubbing the organs between two pieces of 60-mesh stainless-steel gauze. Using siliconized glassware throughout, cells were washed three times in tris(hydroxymethyl)aminomethane (Tris)/ammonium chloride buffer (1 part of Tris buffer containing 20.6 g of Tris per liter of distilled water and adjusted to pH 7.2 to 7.4; nine parts ammonium chloride, 0.91 g/100 ml), and then suspended in bicarbonate-buffered RPMI 1630 medium supplemented with 0.3 mg of glutamine per ml, 0.1 mg of sodium pyruvate per ml, 100 U of penicillin G per ml, 100 μg of streptomycin per ml, and 2% pooled human serum. The human serum was collected locally, filter sterilized, heat inactivated (56 C, 30 min), and stored in aliquots at −70 C; it had <1:10 hemagglutination inhibiting (HI) antibody titers against the two influenza viruses being used and was shown previously to support proliferative responses of mouse node and spleen cells in vitro.

Antithymocyte serum treatment of cells. Rabbit anti-mouse thymocyte serum (ATS) was purchased from Microbiological Associates. This and normal rabbit serum (NRS) were inactivated at 56 C for 30 min and absorbed with CBA bone marrow cells. Guinea pig serum absorbed with CBA node and spleen cells were used as a source of complement. Sera were filter sterilized and frozen in aliquots.

Node and spleen cells were washed in Tris/ammonium chloride buffer, and 1.5×10^6 mononuclear cells in 1.5 ml of medium were incubated with 0.5 ml of a dilution of NRS or ATS for 30 min at 37 C. After centrifugation at $200 \times g$ for 10 min, the cells were resuspended in 2.5 ml of medium plus 0.5 ml of guinea pig serum and again incubated at 37 C for 30 min. The cells were then washed in medium and used for study.

The dilution of NRS and ATS used for these treatments resulted in no loss of CBA thymocytes with NRS when compared to results with medium alone, and 95 to 100% killing with ATS. This treatment of node and spleen cells with NRS resulted in an average 32% loss of viable mononuclear cells, whereas the loss with ATS averaged 52%. Using techniques described by Shortman et al. (11), the ATS treatment resulted in a 78% reduction of the stimulation index of node and spleen cells to phytohemagglutinin and a 6% increase in the stimulation index to pokeweed mitogen when compared to results with NRS-treated cells; these data suggest that the predominant lymphocyte population removed by ATS treatment was phytohemagglutinin-responsive T cells, and that the remaining cells retained responsiveness to the more universal pokeweed mitogen stimulus.

Adoptive immunization. Node and spleen cells contained in 0.2 ml of medium were injected into the tail vein of each previously unsensitized recipient mouse. Twenty-five to 30 million viable (trypan blue exclusion) mononuclear cells were given to each recipient unless otherwise specified; decreasing the number to 20 million obscured the effects to be described. Groups of 10 to 15 recipients of each cell preparation whose effects were to be compared were always prepared and challenged with the same virus inoculum 1 to 2 h later.

Virus challenge. Influenza A PR8 and B Lee were grown in embryonated eggs and stored in aliquots at −70 C. Virus challenge was intranasal under light ether anesthesia with a volume of 0.05 ml. The doses of virus used caused uniform infection as judged by antibody titers on sera from surviving mice and were adjusted for an anticipated 50% total mortality in control mice. When mortality of recipients of control cells was less than 25%, the effect of sensitized cells on mortality was minimized.

Illness was characterized by decreased activity, ruffled fur, and rapid, shallow respirations. Times of death were recorded for construction of survival curves used to compare the effects of materials given recipient mice. Mice who died were autopsied and always had panlobar consolidation of their lungs. These lungs yielded a thin, serosanguinous fluid on excision and characteristically yielded the challenge virus on culture.

RESULTS

Adoptive immunization with cells from donors vaccinated with formalin-inactivated influenza (homologous challenge). Mice adoptively immunized with 25 million syngeneic washed node and spleen cells from donors vaccinated subcutaneously with formalin-inactivated influenza A PR8 had a higher mortality with influenza pneumonia than recipients of similar cells from unsensitized control donors

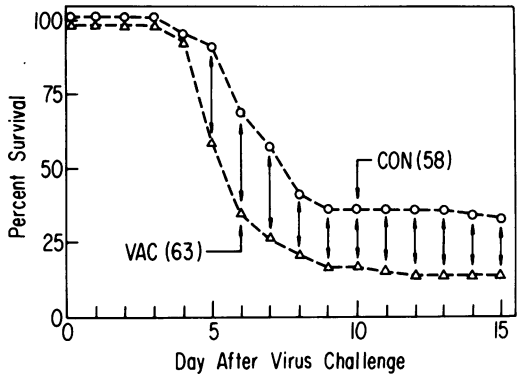

FIG. 1. *Survival curves after intranasal challenge with influenza A PR8 for mice who had been transfused with 25 million washed node and spleen cells from either donors vaccinated subcutaneously with formolized influenza A PR8 (VAC) or unsensitized donors (CON). On all graphs, the numbers in parentheses indicate the number of mice represented by the curve, and vertical lines between the curves indicate that the proportions of surviving mice on that day were significantly different by chi-square analysis ($P < 0.05$).*

after intranasal challenge with influenza A PR8 as shown in Fig. 1. Maximal differences in mortality between the two groups occurred on days 5 to 7 after challenge (chi-square on each day = 9.9 or greater, $P < 0.01$), but differences persisted through the 15-day observation period (final survival, 9/63 for sensitized cell recipients versus 19/58 for control cell recipients, chi-square = 4.8, $P < 0.05$).

Both the washing procedure and the number of node and spleen cells transferred to each recipient mouse were important for producing the results shown in Fig. 1. In earlier work, unwashed node and spleen cells had been adjusted to deliver 10 million mononuclear cells per recipient mouse. This number of unwashed, sensitized cells protected against death with influenza pneumonia after homologous virus challenge (31/64 surviving versus 16/62 with similar preparations of control cells, chi-square = 6.0, $P < 0.05$) rather than causing decreased survival and did not influence mortality after challenge with heterologous influenza B Lee (14/41 surviving versus 17/42 with control cells). HI antibody titers of the unwashed node and spleen cell suspensions were less than 1:10. These results were initially interpreted as indicating protection mediated by the cells (Clin. Res., **19**:78, 1971). However, after being washed in Tris/ammonium chloride buffer to lyse erythrocytes and remove any contaminating antibody, 10 million sensitized node and spleen cells failed to protect (12/39 surviving versus 13/41 with control cells), suggesting that some factor(s) in the unwashed cell suspensions besides the mononuclear cells was needed to mediate the protection. Increasing the number of washed cells per recipient mouse to 25 million caused the higher mortality of recipients of sensitized cells than of control cells after challenge that is shown in Fig. 1.

Serum HI antibody titers of mice who received 25 million washed, sensitized cells and survived challenge with influenza A PR8 were similar to and had the same median titer as those of surviving recipients of 25 million washed control cells.

Effect of antithymocyte serum on results of homologous challenge. Node and spleen cells from donor mice vaccinated subcutaneously with inactivated influenza A PR8 were treated with either NRS or rabbit ATS, and recipient mice were given 25 million viable cells remaining after these treatments. After challenge with influenza A PR8, mortality of recipients of NRS-treated, sensitized cells was high like that of untreated, sensitized cell recipients, whereas mortality of ATS-treated, sensitized cell recipients was reduced and resembled that of control cell recipients (Fig. 2). Thus, ATS treatment appeared to block the detrimental effects of the sensitized cells for the adoptive host after virus challenge (final survival, 13/34 for recipients of ATS-treated, sensitized cells versus 3/30 for recipients of NRS-treated, sensitized cells, chi-square = 5.4, $P < 0.05$).

Heterologous challenge. Recipients of 25 million washed node and spleen cells from control donors and from donors vaccinated

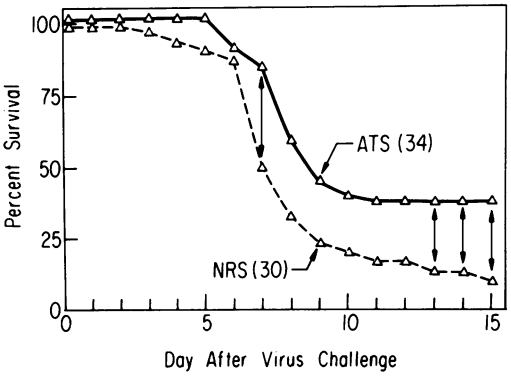

FIG. 2. *Washed node and spleen cells from mice vaccinated subcutaneously with formolized influenza A PR8 were treated with either rabbit ATS or NRS. Survival curves after intranasal challenge with influenza A PR8 are for mice who had been transfused with 25 million viable cells remaining after the above treatments.*

subcutaneously with inactivated influenza A PR8 were challenged with heterologous influenza B Lee. As shown in Fig. 3, recipients of sensitized cells had a lower survival than control cell recipients (final survivals, 43/77 versus 60/79, chi-square = 6.20, $P < 0.05$) suggesting that the detrimental effects of the sensitized cells for the adoptive host lacked antigenic specificity for the influenza challenge virus.

HI antibody titers against influenza B Lee for the surviving recipient mice in each group were similar, and no mice developed antibody to influenza A PR8.

Adoptive immunization with cells from donors vaccinated with typhoid vaccine. Recipients of cells from donors vaccinated subcutaneously with inactivated typhoid bacilli on a schedule similar to that with inactivated influenza A PR8 had a survival curve after challenge with influenza A PR8 (day 6 and final survival, 12/14 and 3/14) that was nearly identical to that of concomitant control cell recipients (day 6 and final survival, 13/15 and 3/15).

Adoptive immunization with cells from previously infected donors. Mice who had received no cells but who had survived two intranasal challenges with live influenza A PR8 were used as donors of node and spleen cells 4 to 6 weeks after the second challenge. Mice adoptively immunized with these cells and challenged with influenza A PR8 had a survival curve (day 6 and final survival, 20/27 and 6/27) very similar to that of concomitant control cell recipients (day 6 and final survival, 16/26 and 6/26) as shown in Fig. 4.

Adoptive immunization with cells from donors vaccinated subcutaneously with live influenza. Donor mice were vaccinated subcutaneously with live influenza A PR8 on a schedule like that with inactivated virus. Mice adoptively immunized with cells from these donors and then challenged with influenza A PR8 had a survival curve (day 6 and final survival, 22/29 and 9/29) very similar to that of concomitant control cell recipients (day 6 and final survival, 21/29 and 12/29) as shown in Fig. 5.

Passive immunization with antibody. Mice injected intravenously with 0.2 ml of mouse antiserum having an HI antibody titer of 1:40 against influenza A PR8 had a survival curve

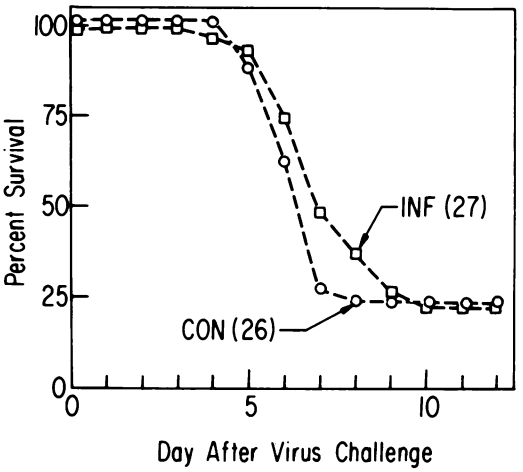

FIG. 4. *Survival curves after intranasal challenge with influenza A PR8 for mice who had been transfused with 25 million washed node and spleen cells from either donors who had survived influenza A PR8 respiratory infection (INF) or unsensitized donors (CON).*

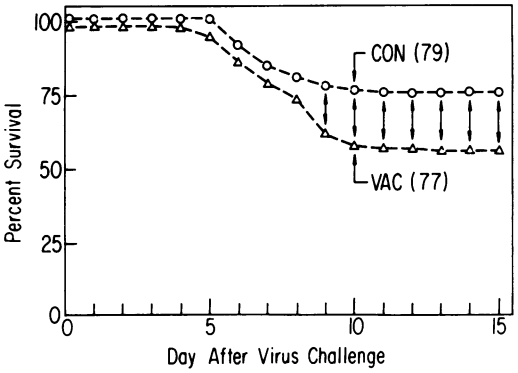

FIG. 3. *Survival curves after intranasal challenge with heterologous influenza B Lee for mice who had been transfused with 25 million washed node and spleen cells from either donors vaccinated subcutaneously with formolized influenza A PR8 (VAC) or unsensitized donors (CON).*

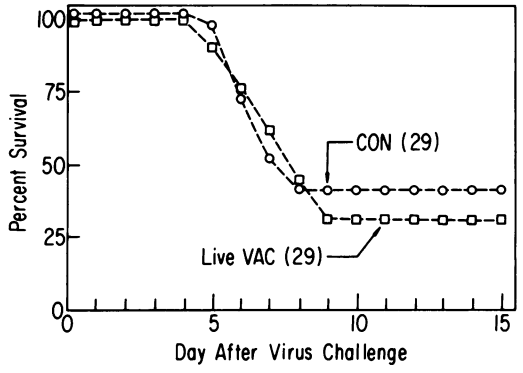

FIG. 5. *Survival curves after intranasal challenge with influenza A PR8 for mice who had been transfused with 25 million washed node and spleen cells from either donors vaccinated subcutaneously with live influenza A PR8 (Live VAC) or unsensitized donors (CON).*

after challenge with this virus (day 6 and final survival, 15/32 and 5/32) very similar to that of simultaneously challenged recipients of 0.2 ml of antibody-free mouse serum (day 6 and final survival, 13/26 and 7/26).

DISCUSSION

These studies indicate that washed node and spleen cells from syngeneic mice vaccinated subcutaneously with formalin-inactivated influenza A PR8 can increase influenza mortality in adoptive hosts after intranasal challenge with homologous virus when compared to results in recipients of unsensitized cells. This increased mortality can be ablated by treatment of the sensitized cells with antithymocyte serum prior to transfer, suggesting that a thymus-dependent cell population is necessary for the effect. That thymus-dependent cells may be important in the pathogenesis of influenza pneumonia, even during primary infection, has been suggested by others on the basis of decreased lung consolidation and mortality after challenge of mice treated with antilymphocyte serum as compared to results with normal rabbit serum (12), though results in such models have been conflicting (5).

Passive immunization with mouse serum containing HI antibody did not alter mortality from that observed with control serum in the present model. Surviving recipients of sensitized and unsensitized cells developed similar serum HI antibody titers against the challenge virus, and PR8-sensitized cell recipients who survived challenge with influenza B Lee developed no detectable antibody against influenza A PR8. Thus, it does not seem likely that circulating HI antibody was involved in causing the increased influenza mortality among recipients of cells from mice vaccinated with formalin-inactivated influenza A PR8. This effect was also not likely due to local antibody formation by the transferred cells since such antibody-forming cells should not have been eliminated preferentially by treatment with antithymocyte serum as compared to normal rabbit serum.

The increased mortality of mice adoptively immunized with cells from donors vaccinated with inactivated influenza A PR8 occurred not only after challenge with homologous virus but also after challenge with heterologous influenza B Lee. Cells from donors vaccinated with typhoid vaccine failed to alter mortality of adoptive hosts after challenge with influenza A PR8, indicating some selectivity of the antigens which can induce the effect. Egg proteins were undoubtedly present in the partially purified, inactivated influenza A PR8 vaccine and in the challenge inocula for both influenza A PR8 and influenza B Lee, but the occurrence of most deaths 5 to 7 days after challenge suggests that viral replication had to occur and that the increased mortality of recipients of sensitized cells involved an interaction with the virus rather than any nonreplicating antigens included in the challenge inoculum. Failure of vaccination with live, egg-grown influenza A PR8 to induce a similar effect (see below) is further evidence against a critical role of egg proteins in producing the results.

Washed node and spleen cells from donors who had survived infections with influenza A PR8 failed to modify survival of adoptive hosts from that observed in recipients of unsensitized cells after challenge with the virus. One possible explanation for the difference in effects of sensitized cells from vaccinated and previously infected donors was that nodes draining sites of subcutaneous vaccination might be harvested more completely than those draining sites of infection in the respiratory tract, and this might have led to a difference in the percentage of sensitized cells among node and spleen cells from the two groups of donors. Studies were therefore undertaken using subcutaneous vaccination with live influenza so that the sampling of nodes draining the site of virus exposure could be identical to that in donors vaccinated with inactivated virus. Washed node and spleen cells from donors vaccinated subcutaneously with live influenza A PR8, like those from previously infected donors, failed to cause a significant increase in influenza pneumonia mortality in adoptive hosts over that observed in unsensitized cell recipients after challenge with homologous virus.

These data reveal a difference in the effect of sensitized node and spleen cells on the course of influenza pneumonia in adoptive hosts depending on whether the sensitizing virus was live or inactivated, and moreover, an increase in influenza mortality in recipients of cells from donors vaccinated with inactivated influenza A PR8 after challenge by either homologous virus or heterologous influenza B Lee. Potentially analogous findings have been reported in other situations. In a study conducted years after the initial virus exposure, Lennon et al. (9) noted that a high proportion of previous recipients of an inactivated paramyxovirus vaccine, measles, exhibited delayed skin reactivity not only to measles antigen but also to poliovirus antigen, whereas such reactivity was negligible or absent in persons who had had live measles vaccine or natural measles; the possiblity was raised that aberrant CMI sensitization might contribute to

the severe and atypical disease sometimes observed in recipients of inactivated measles vaccine when measles infection subsequently occurred. A report from this laboratory (1) described differences in responsiveness of human peripheral lymphocytes to phytohemagglutinin depending on whether the subject had had inactivated Hong Kong influenza vaccine or clinical influenza several months previously, cells from subjects who had had vaccine exhibiting significantly greater responsiveness. The suggestion emerges from these observations that inactivated virus vaccines, particularly for myxo- and paramyxoviruses, induce a CMI responsiveness to homologous virus and other stimuli which differs from that induced by live virus and which may increase the severity of disease when the host subsequently becomes infected.

Based on this analysis, the reported increased severity of naturally occurring RSV disease in young children who had previously been vaccinated with a formalin-inactivated, alum-precipitated trial vaccine made from this virus (7, 8) could have been at least partially or solely accounted for by an aberrant CMI responsiveness induced by the inactivated vaccine. The hypothesis that serum antibody induced by the RSV vaccine might have been important in the pathogenesis of the severe disease during later infection of young vaccinees was based in part on a proposed analogy to the severe RSV disease occurring in infants who can acquire circulating immunoglobulin G antibody transplacentally from their mothers, but not secretory immunoglobulin A antibody nor sensitized cells of the CMI system (2). However, the severe disease observed in infants infected with RSV may be due to some very different factor(s) such as infection of small airways which are already major contributors to total lung resistance at this age (6) and/or lack of nonspecific resistance induced by previous respiratory infections (4). Moreover, at least in a mouse influenza model, passively administered antibody does not augment the severity of disease even when administered after the onset of infection (3).

The severe RSV disease occurred primarily in vaccinees below 2 years of age and not in older vaccinees who were more likely to have had a natural exposure to RSV prior to vaccination (7). If the analogy of this RSV experience to the present mouse influenza model holds, then the form of primary exposure to a given or related virus, inactivated or live, may be critical for determining the type of CMI responsiveness which will develop with forms of secondary exposure having less importance. Thus, one might anticipate that inactivated influenza vaccine administered to older children and adults would not be a strong stimulus of aberrant CMI responsiveness because of previous sensitization by infection with the same virus or one exhibiting cross-reactivity in the CMI system.

Vaccination against respiratory virus disease with live attenuated virus intranasally has an advantage over peripheral vaccination with inactivated virus in more effective induction of secretory antibody. The data presented suggest that differences in effects of these vaccines on the CMI system may provide another equally important rationale for the use of live virus vaccine.

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