Respiratory Tract Cell-Mediated Immunity: Comparison of Primary and Secondary Response

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A secondary local and splenic cell-mediated immune response was observed and compared to the primary response. Previous studies have demonstrated cell-mediated immunity (CMI) by lymphocytes from bronchopulmonary washings and have shown that its appearance is to a large extent independent of splenic CMI. This study evaluated the secondary as compared to the primary response, with respect to both cellular and humoral immune responses. Guinea pigs were immunized with influenza virus vaccine either nasally or parenterally. Booster immunizations were given by the same route, and animals were killed at various times after immunization or booster. The inhibition of macrophage migration was used to assess CMI. As in previous studies, local application of antigen led to mainly local appearance of CMI, whereas parenteral immunization led to mainly systemic CMI. Both pulmonary and splenic lymphocytes showed an inhibition of macrophage migration that appeared 2 to 3 days sooner after the booster, as compared to the primary immunization. There was no evidence, however, for the earlier production or increased amount of antibody in the bronchial secretions in the boosted animals. The results suggest that pulmonary as well as splenic T lymphocytes exhibit memory, but that pulmonary B lymphocytes do not.

Previous studies have demonstrated the presence of cell-mediated immunity (CMI) on secretory surfaces and have shown that local CMI can be stimulated independently of systemic CMI (5, 14, 15). The appearance of CMI in the respiratory tract and spleen is dependent on the route of immunization. Thus, respiratory tract CMI is more effectively stimulated by administration of the antigen into the respiratory tract, whereas splenic CMI is better stimulated by subcutaneous administration.

Using the inhibition of macrophage migration, CMI has previously been evaluated in the respiratory tract mainly with respect to the primary response, although a secondary response has been demonstrated by Nash and Holle (8). It was the purpose of this study to further investigate the secondary response on a secretory surface, i.e., immunological memory in the respiratory tract.

MATERIALS AND METHODS

Guinea pigs. Hartley strain guinea pigs, approximately 400 g in weight, were used for immunization and as a source of peritoneal exudate cells.

Vaccine. The commercially available bivalent influenza virus vaccine (Eli Lilly & Co.) containing 400 CCA units of A/Aichi/2/68 (Hong Kong variant) and 300 CCA units of B/Mass/3/66 per dose was used in a dose of 0.1 ml diluted 1:10 with phosphate-buffered saline, pH 7.4.

Immunization. A total of 140 animals were immunized either by administration of 0.1 ml of a 1:10 dilution of the vaccine by nose drops on 4 successive days or by injection of 0.1 ml of the vaccine mixed with an equal volume of complete Freund adjuvant (H37Ra) into each hind footpad. To determine the secondary response, approximately 45 days after immunization, groups of nasally immunized and parenterally immunized animals were boosted in the same manner.

Collection of specimens. At various times after immunization and boosting, four to six guinea pigs immunized by nose drops or by footpad injections were given a lethal dose of sodium pentobarbital. While the guinea pigs were still living, the thoracic cavity was opened and each animal was exsanguinated by cardiac puncture. The blood was saved, and the serum was separated for use in antibody determinations. The trachea was then clamped as cephalad as possible and cut above the clamp. The respiratory tract was removed, and the exterior was washed with phosphate-buffered saline. The clamp was removed, and pulmonary cells were obtained by alternately instilling into and aspirating from each lung Eagle medium containing 100 µg each of penicillin and streptomycin. A total of approximately 5 ml of
media was instilled into each lung. Cells from pairs of immunized guinea pigs were pooled, and the supernatants from these suspensions were assayed for antibody activity. Although allogenic cells were pooled, a mixed lymphocyte reaction did not appear to contribute to inhibitions, since there was no inhibition when the pooled cells were incubated with medium alone. At the same time, the spleen was removed from each animal and then pressed and washed through a wire mesh screen with Eagle medium. Spleen cells from pairs of immunized animals were pooled. All cell suspensions were then centrifuged at 1,200 rpm for 10 min, washed twice in Eagle medium, resuspended in TC199 containing 5% fetal calf serum, 10% heat-inactivated guinea pig serum, and 100 μg each of penicillin and streptomycin per ml, and adjusted to 25 × 10⁶ cells per ml.

Inhibition of macrophage migration. The method of David (3) was employed. Oil-stimulated peritoneal macrophages were obtained from nonimmunized guinea pigs for use as indicator cells. Half of each pulmonary cell suspension was mixed with normal guinea pig peritoneal exudate cells in a ratio of 1:4, and the other half was mixed in the same ratio with normal peritoneal cells plus dialyzed influenza virus vaccine diluted 1:100 in TC199. Chambers containing capillaries with normal peritoneal cells in media alone or in media plus vaccine were used as controls. Six capillary tubes were filled from each suspension, sealed with paraffin wax, and centrifuged at 800 rpm for 5 min. Each tube was then cut at the cell interface and mounted into Mackaness chambers filled either with TC199 or dialyzed vaccine diluted 1:100 in TC199. After incubation of the chambers at 37°C for 48 h, the migration areas were projected onto paper and traced. The area of migration was measured using a planimeter, and the effect of antigen was expressed by calculating 100% = [(area of migration of cells with antigen)/(area of migration of cells without antigen)] × 100.

Influenza-neutralizing antibody. Antibody titers were measured by the hemadsorption-inhibition neutralization technique previously described (2).

RESULTS

Inhibition of macrophage migration. The results in this study confirm the previous observations that the magnitude of response depends upon the route of immunization. In guinea pigs given nose drops, a much higher response was observed in the lungs (maximal mean inhibition [max MI] = 62% at 21 days) (Fig. 1) than in the splenic cells (max MI = 35% at 6 to 7 days). This difference is significant at less than the 0.01 level. Guinea pigs given footpad injections showed a much higher splenic response (max MI = 54% at 14 days) than pulmonary response (max MI = 29% at 14 days, P < 0.05, using the t test for two sets of data with homogeneous variances).

These findings appear to indicate that stimulation of sensitized lymphocytes in the lung is more effectively produced by local application of the antigen, whereas stimulation in the spleen is more effectively evoked by parenteral administration of antigen.

The booster response was characterized by the earlier appearance of inhibition of macrophage migration (IMM) than the primary response (Fig. 2). In animals immunized by nose drops, no inhibition was found 2 to 3 days after primary immunization in the lungs, but an MI of 25% was seen 2 to 3 days after boosting (P < 0.01). Booster immunizations were carried out 43 to 45 days after the primary. At that time, animals receiving no booster showed no residual inhibition by their pulmonary cells. There was no significant difference in the intensity or duration of the booster as compared to the primary response.

Again, comparing the spleens of guinea pigs immunized subcutaneously, the primary response showed an MI of 3% 2 to 3 days postimmunization, whereas the booster response showed an MI of 24% after 2 to 3 days (P < 0.01). And again, the primary response in the spleen had disappeared by 45 days, the time of the boosting. The intensity and duration of both responses were the same.
Humoral antibody. Production of influenza-neutralizing antibody in the bronchial washings and serum of both locally and systemically immunized animals was dependent on route of immunization (Fig. 3). Antibody in bronchial washings was found only after local immunization. Similarly, the mean maximal serum-neutralizing antibody in guinea pigs given footpad injections was 1:64, whereas that in nasally immunized animals was 1:6. After primary immunization, antibody was first detected at 6 to 7 days in the bronchial washings of nasally immunized animals, whereas after booster immunization, antibody was detectable at 2 to 3 days. However, the mean antibody titer was no higher than that remaining in the guinea pigs 45 days after the primary immunization, so this cannot be taken as evidence for a memory system. In addition, the primary and secondary responses were not of statistically different intensity in the bronchial washings of guinea pigs given nose drops (Fig. 4).

In the bronchial washings of guinea pigs given footpad injections, no antibody was detectable after primary immunization, whereas neutralizing antibody was found, although at low titers, after the booster (differences were not statistically significant).

DISCUSSION

The results of this study confirm previous findings from this laboratory that CMI and antibody production in the respiratory tract and systemically are dependent on the route of immunization. When antigen was administered locally by nose drops, a significantly greater CMI response was observed in the animals' lungs than in their spleens, and conversely, when the antigen was given systemically there was a much greater response in spleens than in lungs. The lack of correlation between lung and spleen responses indicates that local and systemic CMI are, for the most part, independent.

Similar results have been obtained in other recent studies of CMI in the respiratory tract. Galindo and Myrvik (4) showed that CMI induction in the respiratory tract was independent of systemic CMI and that it varied with the...
route of immunization. Rabbits immunized subcutaneously with BCG developed dermal tuberculin sensitivity, whereas rabbits immunized intravenously did not. Alveolar cells from subcutaneously immunized rabbits showed little inhibition by mycobacterial antigens in the migration inhibition test, whereas alveolar cells from rabbits immunized intravenously were consistently inhibited by these antigens. Kawai et al. (6) showed that alveolar wash cells from rabbits immunized intratracheally with *Mycoplasma faeni* were able to inhibit macrophage migration in the presence of antigen.

Although local production of CMI in the respiratory tract has been established (15), there are few reports regarding a secondary CMI response on secretory surfaces. Kawata et al. showed that rabbits previously immunized with BCG undergo an accelerated macrophagic granulomatous response after challenge (7). It is generally accepted that a secondary response represents the lymphocyte’s capacity to exhibit memory, defined as a state of heightened reactivity to a previous antigenic contact (11). We have interpreted an earlier appearance of CMI in the respiratory tract and spleen as representing the capacity of T lymphocytes for memory. This could be due either to sensitized T lymphocytes produced locally in the lung or to sensitized lymphocytes migrating to the lungs, possibly related to the inflammation set up in response to the antigen. Studies to differentiate between these alternatives are underway, but the time course of the response favors the former explanation, because any inflammation after administration of killed influenza virus would be very short-lived. Thus, memory seems to be exhibited by both systemic and secretory T lymphocytes.

T lymphocyte memory in the humoral response has been extensively studied. Although it has been shown that T cells involved in the enhancement of antibody production have the capacity for memory (10, 13), it is not known whether these T cells are the same T cells involved in CMI.

Systemic memory in T lymphocytes active in CMI has previously been demonstrated in second-set graft rejections, a presumed function of CMI (12). However, it has been difficult to demonstrate directly T lymphocyte memory because of the unavailability of suitable in vitro tests.

The development of the IMM test has provided a method. Nash and Holle (8) have shown a secondary response in bronchial washing, spleen, and in peritoneal exudate cells after secondary low-dose local stimulation with 2,4-dinitrophenol-HGG. Cells of all three populations did not inhibit migration of macrophages until 1 week after primary immunization but inhibited migration 4 days after secondary challenge, memory being indicated by the earlier appearance of IMM. Similarly, we have shown that the appearance of IMM is more rapid in both bronchial washing cells from locally boosted animals and spleen cells from parenterally boosted animals. Neither a heightened nor a more prolonged response was observed. The earlier appearance of CMI in the lower respiratory tract after the second exposure to the antigen may be important in protection against infections of this secretory surface. When influenza virus infection occurs, the difference of few days between the onset of CMI in the sensitized and nonsensitized individual might be a determining factor in whether clinical disease occurs or not. Both primary and secondary local CMI was best stimulated by nose-drop immunization.

Antibody did not appear earlier and was not detected in higher titer in the bronchial washings of guinea pigs given either nose drops or footpad injections, suggesting that secretory B lymphocytes have no capacity for memory. This
agrees with a study in humans showing no evidence of a heightened or earlier response after secondary local administration of inactivated poliovirus vaccine in the nasopharynx (9).

In contrast, another study suggested the presence of respiratory tract memory, because a nonimmunizing dose of inactivated rhinovirus vaccine given intranasally elicited a significant local antibody response in men previously infected or vaccinated (1). It is possible that the different results depend on the antigen used, or in the timing of the booster immunization. It is possible that a longer interval between the primary and secondary immunization might have resulted in an evident memory response.

Local administration of antigen produced neutralizing antibody in both bronchial washings and serum, whereas parenteral immunization caused a high serum-neutralizing antibody response but very little antibody production in bronchial washings. Thus, although systemic introduction of antigen initiates both a high CMI response in the spleen and high antibody titers in the serum, there is poor stimulation of both CMI and antibody production in the respiratory tract—possibly important mechanisms in host defenses against viral infections of the respiratory tract.

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LITERATURE CITED