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Suppression of Tuberculin Hypersensitivity During Influenza Infection in Mice

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Tuberculin hypersensitivity was examined during acute influenza infection in mice. Employing the footpad test as a measure of delayed-type hypersensitivity, it was noted that tuberculin hypersensitivity was suppressed temporarily beginning on day 3 and continuing through days 10 to 16 following intranasal infection with influenza A/PR8. These changes occurred at a time when influenza virus was replicating in lung tissue. Suppression of footpad swelling was not detected when mice were administered live virus intravenously, were given Formalin-inactivated virus intranasally, or were immunized against influenza before intranasal infection. Transient reduction of total circulating lymphocytes also occurred during influenza infection but did not correlate with the duration of footpad suppression. Because this model system reproduces many of the alterations in immunological function reported to occur during influenza infection in humans, it should provide a useful tool for investigating mechanisms of influenza-induced immunosuppression.

Temporary suppression of delayed-type hypersensitivity (DTH) has been observed during several acute viral infections of humans (6, 10, 14). One of the earliest descriptions of this phenomenon was a report by Bloomfield and Mateer (1) that tuberculin hypersensitivity was suppressed in patients with an acute influenza-like syndrome. These early findings were subsequently confirmed by Reed et al. (16), who examined cellular immune responses in patients with documented influenza virus infection. Employing in vitro correlates of cell-mediated immunity, several investigators have also shown that mitogen-induced lymphocyte proliferation was reduced during the acute and convalescent phases of the illness (2, 7, 9). These alterations in immune function were often accompanied by transient reduction of circulating lymphocytes. Whether immunosuppression which accompanies influenza infection is of any consequence to the host is still uncertain. It has been suggested that these changes, even though temporary, may predispose the host to concomitant infection with other facultative intracellular parasites (21). In support of this claim, Volkert et al. (22) reported that tuberculosis was exacerbated in mice simultaneously infected with influenza virus. It has also been suggested that temporary suppression of cell-mediated immunity may predispose the host to neoplastic diseases (3, 7). It seems important, therefore, to elucidate and further define the mechanisms of influenza-induced immunosuppression. Previous attempts to reproduce and examine this phenomenon in animals have been unsuccessful (11). Since influenza virus produces only a self-limited infection in the upper airways in guinea pigs and ferrets, it was suggested that influenza-induced immunosuppression may depend upon the capacity of the virus to replicate in lung parenchyma (11).

This report describes suppression of tuberculin hypersensitivity during influenza infection in mice. The $R1R_v$ attenuated strain of *Mycobacterium tuberculosis* (19) produces a nonlethal infection in mice. Gray and Jennings (4) have shown that injection of tuberculin antigen into the footpads of immunized mice will produce delayed swelling which mimics delayed hypersensitivity in humans, and that the immunological response can be quantitated by determining the extent of the swelling. Using this method to monitor tuberculin hypersensitivity, we were able to show that footpad swelling was temporarily suppressed during acute influenza infection in mice and at a time when the virus was replicating in lung parenchyma. This animal model system should prove useful in elucidating mechanisms of influenze-induced immunosuppression.

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MATERIALS AND METHODS

Animals. Eight- to 10-week-old random-bred female CF-1 mice (Carworth Farms, Portage, Mich.) were housed in metal cages and offered water and food pellets ad libitum. Infected animals were housed separately in special designated quarters.

Viruses. Influenza A/PR/8/34 (H_0N_1) was obtained from S. Rabinovich, Southern Illinois University, Springfield, Ill., and a virus pool was prepared from a single passage into the chorioallantoic fluid of 11-day-old embryonated hen's eggs. The chorioallantoic fluid was harvested 48 h after infection, clarified by centrifugation, and stored at −70°C. This pool contained $10^{10.2}$ 50% egg infective doses per ml, and the 50% mouse lethal dose (MLD_{50}) was $10^{5.9}$/ml when administered intranasally (i.n.). Influenza virus, inactivated by incubating in 0.05% Formalin at 37°C for 12 h, contained 160 hemagglutinating units per ml and was noninfectious for eggs. The Reed-Muench method (15) was used to determine all 50% endpoints.

Virus infection. Mice were infected by inoculating influenza virus in 0.05-ml portions diluted in phosphate-buffered saline, i.n. under light ether anesthesia. Animals infected via the intravenous (i.v.) route received 0.1 ml of an appropriate dilution of virus by tail vein injection. Uninfected controls were inoculated in the same site with an equivalent amount of normal chorioallantoic fluid or phosphate-buffered saline. Titers of influenza virus in mouse tissues were determined by preparing 10% (wt/vol) suspensions of the tissue in Hanks balanced salt solution, filtering serial 10-fold dilutions of the suspension through 0.45-μm filters, and inoculating 0.2 ml of the filtrate into 11-day-old embryonated eggs. Each dilution was examined in triplicate. The chorioallantoic fluid was harvested at 48 h and examined for hemagglutination with fresh, washed guinea pig erythrocytes.

Hemagglutination inhibition antibody. Mice were bled by cardiac puncture, and the serum was stored at −20°C. The serum was treated with receptor-destroying enzyme (Microbiological Associates) and examined for hemagglutination inhibition antibodies against influenza A/PR8 by using a standard microtiter assay (18).

Immunization. The attenuated $R1R_v$ strain of *M. tuberculosis,* kindly supplied by John Kasik, University of Iowa, Iowa City, Iowa, was grown as a surface culture on Proskauer and Beck medium. Before immunization, the organism was subcultured in Dubos medium, and mice were inoculated i.v. with 0.2-ml samples of the broth culture, containing approximately 10^7 viable units of *M. tuberculosis. Listeria monocytogenes* was maintained by serial passage in mice. The MLD_{50} for CF-1 mice was 10^4 colony-forming units of an overnight growth in Trypticase soy broth. Animals were immunized by inoculating approximately 3×10^3 colony-forming units of *Listeria* i.v. via the tail vein.

Quantitation of the footpad response. Tuberculin-sensitized mice were tested for DTH 4 to 6 weeks after infection, by using the footpad response (4). Twenty-four hours after injecting 0.05 ml of purified protein derivative (PPD; 250 U.S. units/0.1 ml, obtained from Connaught Company, Canada, lot no. 5031-13) intradermally in the right hind footpad, footpad swelling was quantitated by determining the difference in diameter between the injected and contralateral uninjected hind foot using a dial calipers (accurate to 0.05 mm). Footpad swelling that exceeded 1 mm was arbitrarily designated a "positive" response, and only positive animals were employed in subsequent experiments. Positive footpad responses could be elicited reproducibly with each subsequent antigenic challenge. Sequential measurements of the footpad diameter by a single observer revealed a variability of ±0.1 mm.

Mice immunized with *L. monocytogenes* were examined for DTH in a similar manner using an antigen extract kindly supplied by E. Pesanti, University of Iowa, Iowa City, Iowa. The antigen was prepared from an overnight growth of *L. monocytogenes* in Trypticase soy broth. The organisms were sedimented and sonicated, and the protein was precipitated with saturated ammonium sulfate. The precipitate was dissolved in phosphate-buffered saline to a concentration of 100 μg of protein per 0.05 ml. A commercial *Candida* antigen preparation (Dermatophyton O; lot no. C869051, Hollister-Stier Labs) was employed as an additional control.

Suppression of DTH was determined by randomly allocating immunized mice with positive footpad responses into experimental groups of five to seven animals. At selected times after viral infection, the footpad response was determined as previously described. Means and standard deviations for each experimental group were determined and compared with similar measurements from uninfected controls examined simultaneously. Student's *t* test was used for statistical analysis of the data.

Circulating lymphocytes. Peripheral leukocyte counts were performed using a hemacytometer. Differentials were performed manually on smears obtained simultaneously and stained with Wright stain. Circulating lymphocytes were determined indirectly by calculating the fraction of lymphocytes per cubic millimeter.

Histopathology. Histopathological studies of mouse lungs and footpads were performed by fixing the tissue in 10% Formalin, embedding in paraffin, sectioning at 5 μm, and staining with hematoxylin-eosin.

RESULTS

Characterization of the footpad response. The footpad response was employed as a measure of delayed hypersensitivity in the experiments to be described. To assure immunological specificity of this immune response, mice were immunized with *L. monocytogenes* or *M. tuberculosis* and the footpads of immunized animals were injected with either homologous or heterologous antigens. Significant swelling of the footpads ($P < 0.01$) occurred only in immunized animals challenged with a homologous antigen (Fig. 1). Delayed evolution of footpad swelling after intradermal injection of 125 U.S. units of PPD is illustrated in Fig. 2. Swelling was first detected 6 h after injection, peaked at 24 h,

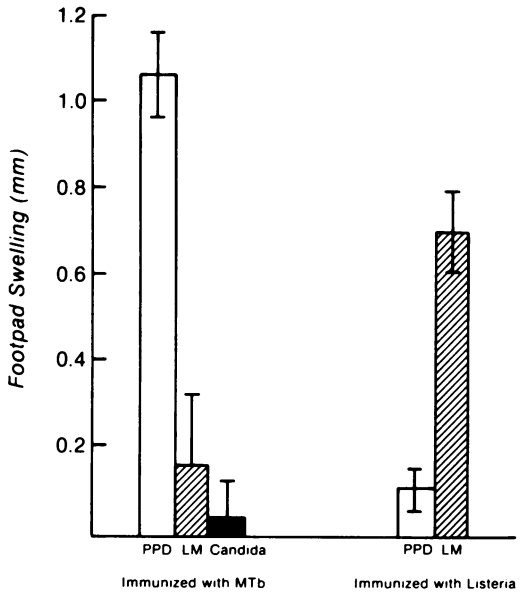

FIG. 1. *Specificity of the footpad response in mice immunized with M. tuberculosis or L. monocytogenes and challenged with homologous and heterologous antigens. Bars represent mean ± standard error of the mean.*

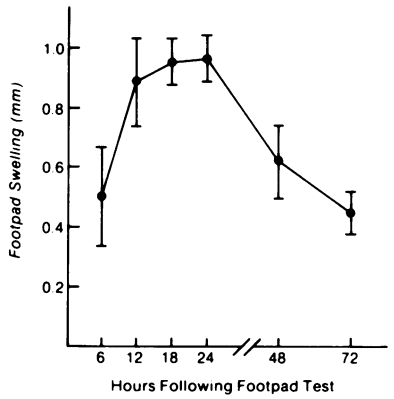

FIG. 2. *Delayed swelling of the footpad in tuberculin-immunized mice after inoculation with PPD (125 U.S. units). Bars represent mean ± standard error of the mean.*

and persisted for 72 h. Histological examination of the footpads of several animals 24 h after injection revealed infiltrates of mononuclear cells in the subcutaneous and underlying fibrous tissues (Fig. 3).

Suppression of the footpad response during influenza infection. Five days after i.n. infection with influenza A/PR8 (1 MLD_{50}), CF-1 mice developed clinical signs consisting of weight loss, ruffled fur, lethargy, and tachypnea. Histological examination of the lungs at this time revealed thickened alveolar walls with infiltrates of mononuclear cells and alveolar exudates. Replication of influenza virus in the lungs of mice infected with 1 MLD_{50} was evident by 24 h and peaked at 5 days, and the virus finally cleared by 17 days postinfection (Fig. 4). Each point on the curve represents the mean 50% egg infective dose per lung of three or four animals.

When tuberculin-immunized mice were infected with influenza A/PR8 (1 MLD_{50}), the footpad response to PPD was temporarily suppressed (Fig. 4). Significant suppression of DTH ($P < 0.01$) was first noted on day 3 after influenza infection and persisted through day 10. Whereas suppression was still detected on day 16 ($P < 0.02$) in one experiment, recovery of a normal footpad response was noted by day 12 in another, suggesting that the duration of immunosuppression may vary somewhat from experiment to experiment. Suppression of the footpad response regularly preceded onset of clinical signs of influenza infection. Because each point on the curve summarizing footpad suppression in Fig. 4 represents a separate group of animals, footpad tests were also examined serially in a few animals during influenza infection. Results confirmed that animals which exhibited suppression of the footpad response during influenza infection resumed a normal response after resolution of the viral infection.

Circulating leukocytes were monitored serially in seven to nine animals from each of two experiments (Fig. 4). Although the total number of circulating leukocytes was unchanged, there was a statistically significant reduction ($P < 0.01$) in the number of circulating lymphocytes on days 2, 5, and 7 postinfection. The results are expressed as percent reduction of the mean total circulating lymphocytes when compared with control values obtained before influenza infection.

Because mortality exceeded 50% in some of the experiments, it seemed important to assure that suppression of the footpad response was not simply a manifestation of impending death. Results from two experiments in which footpad responses to PPD were examined in mice infected with less than 1 MLD_{50} of influenza virus are summarized in Table 1. These results were obtained 6 days after infection, when suppression of footpad swelling was presumed to be maximal. Immunosuppression was noted even at the lowest dilutions, where only an occasional animal died of infection and most appeared clinically well.

Administration of influenza virus i.v. in doses 100 to 1,000 times greater than that given i.n. produced neither clinical signs of disease nor

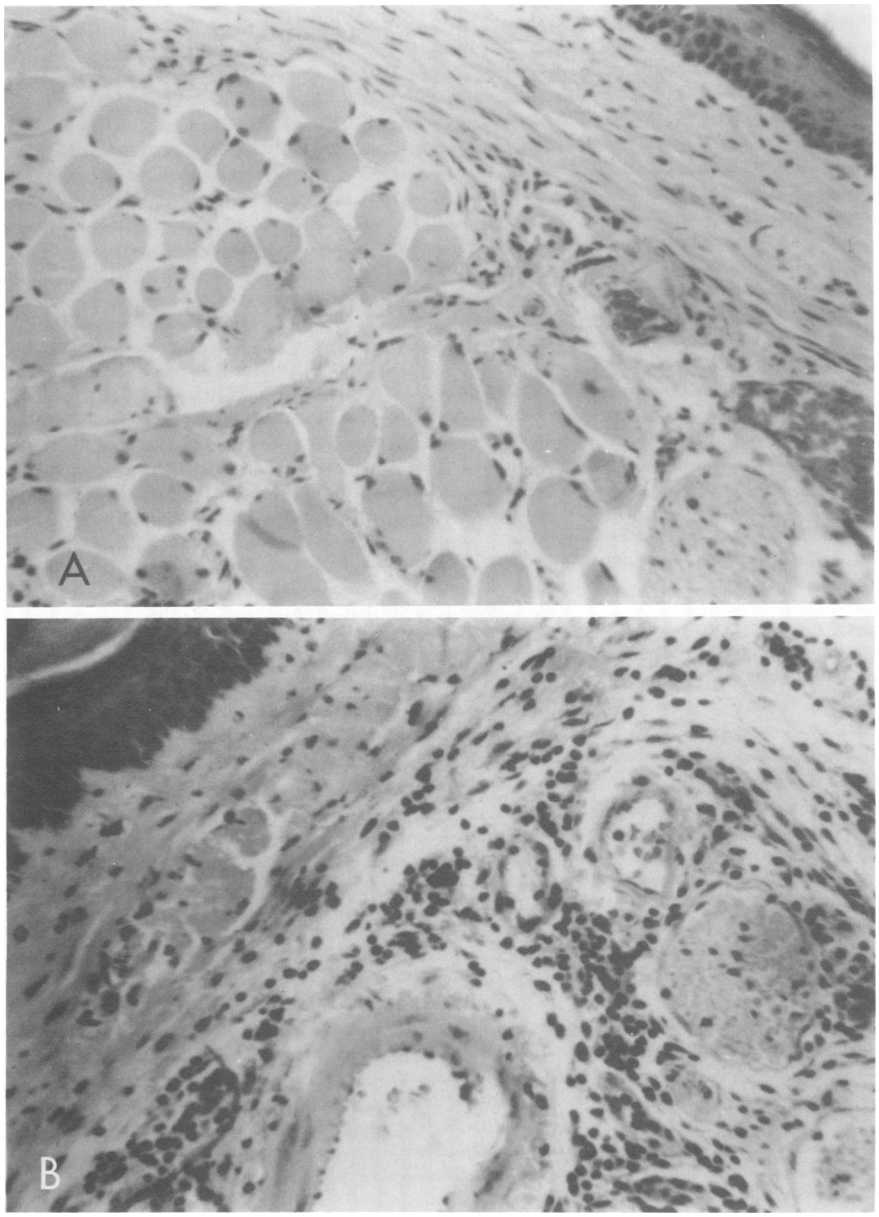

FIG. 3. *Histological sections comparing a normal footpad (A) with that of a tuberculin-immunized mouse 24 h after PPD injection (B). (Magnification, ×160).*

suppression of the footpad test (Table 1). Whereas Table 1 summarizes results obtained 6 days postinjection, the footpad test was also examined at 8, 24, and 72 h after i.v. administration; in no instance was there evidence of suppression. Although influenza virus could not be isolated from the lungs of these animals 6 days after infection, serum influenza hemagglutination inhibition titers of 80 to 1,280 in five of five animals tested indicated that the animals had developed an immunological response to the virus.

Animals infected i.v. with 100 MLD_{50} of influenza A/PR8 were immune to subsequent i.n. infection. When immunized mice were challenged i.n. with 100 to 1,000 MLD_{50} of influenza virus, suppression of the footpad test could not be elicited (Table 1). Finally, when mice were

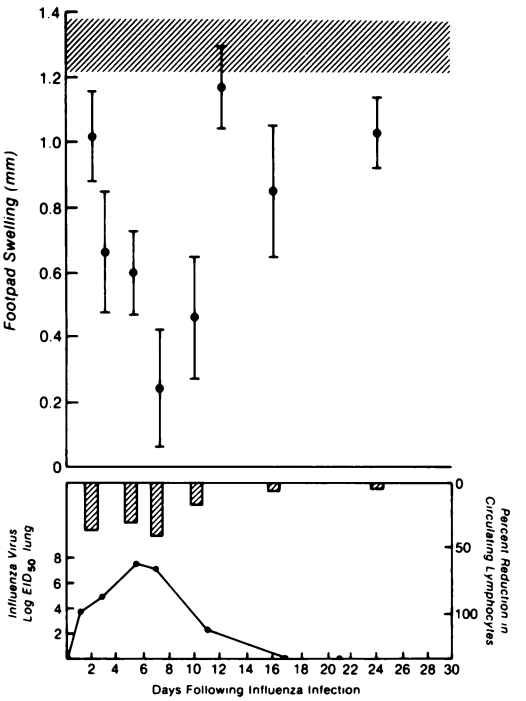

FIG. 4. *Suppression of tuberculin hypersensitivity after i.n. infection with influenza virus. Groups of animals were tested for DTH at various times post-infection (●). Footpad swelling (mean ± standard error of the mean) among control animals is designated by the hatched lines in the upper portion of the figure. Hatched bars in the lower portion of the figure indicate percent reduction of circulating lymphocytes. The curve at the bottom of the figure indicates the titer of influenza virus in mouse lung tissue.*

injected i.n. or subcutaneously with a quantity of Formalin-inactivated virus equivalent to 100 MLD_{50}, footpad suppression did not occur (Table 1).

TABLE 1. *Effect of dose, route of administration, and inactivation of influenza virus on tuberculin hypersensitivity*

Influenza dose (MLD_{50})	Route of administration	No. of animals	Footpad swelling ± SEM[a] (mm)	*P* value
1	i.n.	9	0.64 ± 0.16	<0.01
0.5	i.n.	12	0.55 ± 0.10	<0.01
0.25	i.n.	12	0.67 ± 0.14	<0.01
0.12	i.n.	12	0.55 ± 0.09	<0.01
PBS[b]	i.n.	11	1.58 ± 0.25	
100	i.v.	7	1.16 ± 0.21	NS[c]
1,000	i.v.	7	1.18 ± 0.19	NS
100	i.n.	10[d]	1.36 ± 0.18	NS
100[e]	i.n.	22	1.35 ± 0.10	NS
100[e]	s.c.[f]	23	1.29 ± 0.09	NS
PBS	i.n.	17	1.20 ± 0.14	

[a] Six days after influenza infection. SEM, Standard error of the mean.
[b] PBS, Sterile phosphate-buffered saline.
[c] NS, Not significant.
[d] Mice previously immunized against influenza A/PR8.
[e] Formalin-inactivated virus. Dose equivalents are based on hemagglutination titers.
[f] s.c., Subcutaneous.

DISCUSSION

The pathogenesis of lymphocytopenia and impaired immune responses accompanying influenza infections is still uncertain. Attempts to elucidate these mechanisms through clinical experimentation during influenza infection of humans are necessarily limited. Therefore, an animal model in which this phenomenon can be reproduced should be useful in further investigating mechanisms of virus-induced immunosuppression. Chronic infection of mice with the $R1R_v$ attenuated strain of *M. tuberculosis* (19) afforded a unique opportunity to examine tuberculin hypersensitivity during superimposed viral infection. Marked footpad swelling followed injection of PPD in tuberculin-immunized mice, a response noted by Kasik and Thompson (8) using a similar model system, as well as by Gray and Jennings (4) employing a different strain of *M. tuberculosis*. The footpad response was immunologically specific, delayed in evolution, and marked by infiltrates of mononuclear cells. These characteristics suggested that the footpad test provided a reasonable facsimile of DTH in humans. Ultimate proof for this assumption would depend upon transfer of the immunological response from immunized donors to unimmunized recipients by thymus-derived lymphocytes. Random breeding of CF-1 mice makes such experiments impractical; however, experiments by other investigators have provided substantial evidence to support the assumption that the footpad test is a measure of DTH (13).

Employing the footpad test as a measure of DTH, we demonstrated that mice infected with mouse-adapted influenza A/PR8 exhibited suppression of tuberculin hypersensitivity, reproducing in several respects the phenomenon that occurs in humans. First, there was temporary suppression of the footpad test, which persisted for 10 to 16 days during influenza infection. Similarly, temporary suppression of cellular immune responses has been observed in humans after influenza infection (2, 7, 16). Second, suppression of the footpad test occurred only when mice were infected i.n. with live virus. Animals infected i.v. or inoculated i.n. with Formalin-inactivated virus exhibited no immunosuppression. Since injection of influenza virus via the

i.v. route may not result in viral replication, or at most only mild subclinical disease (17), immunosuppression appears to accompany only more extensive clinical infections, e.g. pneumonitis. A similar association between influenza pneumonitis and altered immunological function has been reported in humans (9). Finally, there was a transient reduction in the number of circulating lymphocytes during influenza infection of mice, an observation which parallels that seen in humans (2). Thus, our model of influenza-induced suppression of tuberculin hypersensitivity in mice shares several features with its human counterpart.

To our knowledge, there has been only one previous report of influenza-induced immunosuppression in animals. Kavetsky et al. (12) reported suppression of antibody formation to a thymus-dependent antigen and suppression of the mixed lymphocyte reaction when mice were infected with influenza A/PR8. In this report, suppression of the mixed lymphocyte response was long-lasting, persisting through 28 days postinfection. In contradistinction to mice, ferrets and guinea pigs exhibited no suppression of cellular immunity during influenza infection (11). The explanation for this disparity may depend upon the extent of the influenza infection in the different animal species. Whereas mice systematically develop pneumonitis with viral replication in lung tissue, influenza infections in ferrets and guinea pigs are usually confined to the upper respiratory tract (5, 20). These observations in animals concur with results reported by Kauffman and co-workers (9), who found that suppression of cellular immunity during influenza virus infection in humans occurred most consistently when the infection involved the lung parenchyma.

Although the pathogenesis of lymphocytopenia and impaired immune responses in this system is still uncertain, several observations deserve comment in this regard. It has been suggested that depletion of circulating lymphocytes may result from alterations of the lymphocyte surface by influenza virus, thereby impairing normal homing properties of the circulating cells (24). This in turn may result in failure of antigen recognition and/or recall following an antigenic challenge (2, 23). In the mouse-influenza model, the duration of lymphocytopenia does not correlate with the duration of the suppressed footpad test. While it is recognized that observations of circulating lymphocytes provide, at best, an insensitive monitor of the homing properties of lymphocytes, these results suggest that additional mechanisms must be postulated to account for the suppression of tuberculin hypersensitivity observed in this system. Studies are currently underway to elucidate mechanisms of immunosuppression during influenza infection in mice and to determine whether this transient impairment in immune function alters the susceptibility of the host to infection with intracellular parasites.

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