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Influenza in Ferrets and Guinea Pigs: Effect on Cell-Mediated Immunity

CAROL A. KAUFFMAN,†* GILBERT M. SCHIFF,[1] AND JOHN P. PHAIR††

Division of Infectious Diseases, Department of Internal Medicine, Cincinnati Veterans Administration Hospital, Cincinnati, Ohio 45220, and the University of Cincinnati Medical Center,[1] Cincinnati, Ohio 45267

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Two different animal models were studied to determine whether localized upper respiratory tract viral infection was associated with suppression of systemic cell-mediated immunity. During influenza infection in ferrets, there was no significant decrease in lymphocyte responsiveness to phytohemagglutinin (PHA). Guinea pigs given influenza showed no significant change in their response to PHA or to picryl human serum albumin (picHSA), to which they had been immunized previously. Delayed hypersensitivity skin test responses to picHSA in guinea pigs remained intact during infection. No change in the percentage of circulating T lymphocytes was detected during influenza infection. Transfer of immunity to nonsensitized recipient guinea pigs from picHSA-sensitized guinea pigs was accomplished during influenza infection. Lack of a suppressive effect on systemic cell-mediated immunity after influenza challenge in these two animal models of mild influenza confirmed previous findings in humans with mild influenza infection.

Viral-induced suppression of systemic cell-mediated immunity is a well-recognized phenomenon (14, 29). Although immunosuppression is rather consistently obtained in humans with viral infections such as measles (24), the question of whether influenza in humans is immunosuppressive is controversial. While some investigators have described diminished lymphocyte transformation during influenza (9, 10), others have failed to find such immunosuppression (11, 20). Skin test anergy during influenza has been described by several investigators (1, 9, 10, 20). Our previous studies in humans showed that the extent of immunosuppression varied with the severity of the infection. Thus, severe influenza pneumonitis was associated with suppression of multiple parameters of cell-mediated immunity (10), but mild influenza localized to the upper respiratory tract did not suppress systemic cell-mediated immunity (11).

We sought confirmation of our studies showing that localized upper respiratory tract viral infections did not lead to a suppression of systemic cell-mediated immunity by studying two different animal models of this type of infection: the ferret influenza and guinea pig influenza models. The pathogenesis of influenza in ferrets is very similar to that in humans with mild influenza (6, 7, 13). Intranasal inoculation leads to local replication of virus in the upper airways, which is only rarely accompanied by pneumonitis (6, 7). The illness is characterized by an acute febrile response, lethargy, and coryza (17, 27). Guinea pigs inoculated intranasally with influenza show viral replication in the anterior nares and develop systemic immunity to influenza, but do not have overt clinical illness (16, 25, 28).

† Present address: Infectious Disease Service, Veterans Administration Hospital, Ann Arbor, MI 48105.

†† Present address: Division of Infectious Diseases, Northwestern university Medical School, Chicago, IL 60611.

MATERIALS AND METHODS

Animals. Adult ferrets were obtained from Marshall Research Animals, Inc., North Rose, N.Y. Adult Hartley guinea pigs and adult strain 13 guinea pigs were purchased from Dutchland Laboratories, Inc., Denver, Pa. Animals receiving viral challenge were housed in a separate room from control animals. All blood samples were obtained by cardiac puncture under ether anesthesia.

Virus pool. The influenza A/Hong Kong/8/68 (H_3N_2) pool was prepared by intra-allantoic inoculation of 10-day-old embryonated eggs with seed virus obtained from Walter Dowdle (Center for Disease Control, Atlanta, Ga.). The titer of the pool was $10^{5.5}$ 50% tissue culture infective doses per ml. The influenza A/PR/8/34 (H_0N_1) pool was prepared in primary rhesus monkey kidney cell culture (Flow Laboratories, Inc., Rockville, Md.) with a seed virus originally obtained from Marion Coleman (Center for Disease Control, Atlanta, Ga.). The titer of the pool was $10^{5.5}$ 50% tissue culture infective doses per ml.

Nasal washes and lung isolation. Nasal washes were performed with 2 ml of phosphate-buffered saline

containing 2% bovine serum albumin, 250 U of penicillin G per ml, and 200 μg of streptomycin per ml; the washes were inoculated into primary rhesus monkey kidney cell cultures. Lungs were removed aseptically, minced, macerated with a pestle, and suspended in 1 ml of Eagle minimum essential medium. A 0.1-ml portion of this suspension was inoculated into primary rhesus monkey kidney cell culture. Each culture was checked for the presence of virus by hemadsorption with a 2% suspension of guinea pig erythrocytes (4).

Hemagglutination inhibition antibody. After overnight incubation with 4 U of *Vibrio cholerae* neuraminidase, the ferret serum was tested for hemagglutination inhibition antibody by the method of Potter et al. (17).

Immunization. Picryl human serum albumin (picHSA) was prepared as described previously (15). Guinea pigs were immunized by footpad inoculation with 100 μg of picHSA in complete Freund adjuvant 3 weeks before viral challenge.

Experimental infection: ferrets. Eighteen ferrets were inoculated intranasally with $10^{5.0}$ 50% tissue culture infective doses of influenza A, Hong Kong, in 0.5 ml of phosphate-buffered saline with 2% bovine serum albumin after light ether anesthesia; eight ferrets received no viral challenge and served as controls. Because of possible differences among influenza strains in virulence for ferrets, a similar but smaller experiment was undertaken with the PR8 strain of influenza. In this experiment, six ferrets were inoculated with $10^{5.0}$ 50% tissue culture infective doses of influenza A, PR8, in 0.5 ml of phosphate-buffered saline with 2% bovine serum albumin after light ether anesthesia; four other ferrets served as controls.

Animals were evaluated twice daily for signs of illness (lethargy, coryza, and rectal temperature elevation). Nasal washes were obtained on day 3 after viral inoculation. Hemagglutination inhibition antibody was assayed in ferret sera collected before and 15 days after viral inoculation; the sera were paired and tested simultaneously.

Two infected animals from each group were sacrificed on day 3 at the height of the illness; lung tissue was examined histologically for evidence of pneumonitis and cultured for virus. The remaining infected and control animals were tested for their response to phytohemagglutinin (PHA), a T-cell mitogen in ferrets (C. Kauffman, unpublished data), 7 days before viral inoculation, on day 3 at the height of the illness, and on day 15 after convalescence.

Experimental infection: guinea pigs. Twenty-four Hartley guinea pigs previously immunized to picHSA received $10^{4.0}$ 50% tissue culture infective doses of influenza A, Hong Kong in 0.5 ml of phosphate-buffered saline with 2% bovine serum albumin intranasally without prior anesthesia; 12 guinea pigs received no viral challenge and served as controls.

Animals were evaluated twice daily for signs of illness (lethargy, coryza, and rectal temperature elevation). Nasal washes were performed on day 3 after viral inoculation (day 2 in the group of animals sacrificed on that day). Antibody titers were not measured because all animals were sacrificed by day 5. Previous studies have indicated that hemagglutination inhibition titers are not measurable at day 7 after inoculation; by day 14, antibody is uniformly present in animals that have excreted virus from the anterior nares (J. P. Phair, C. A. Kaufman, R. Jennings, and C. W. Potter, submitted for publication).

The tests for cell-mediated immunity in guinea pigs included lymphocyte transformation to PHA and picHSA and enumeration of T-lymphocyte numbers. Assays were performed in all animals before viral inoculation and in groups of 12 animals (eight infected and four control) on days 2, 3, and 5 after inoculation.

A second group of eight Hartley guinea pigs previously immunized to picHSA was given influenza A, Hong Kong, as described above and studied with respect to skin test reactivity to picHSA on day 2 after viral challenge. Seven other immunized guinea pigs received no viral challenge and served as controls.

A third group of eight nonimmunized strain 13 guinea pigs was injected intraperitoneally with 10^8 sensitized spleen cells obtained from picHSA-immunized strain 13 donor guinea pigs. The day after cell transfer, the recipients were inoculated intranasally with $10^{4.0}$ 50% tissue culture infective doses of influenza A, Hong Kong, virus. Skin tests with picHSA were performed 48 h later.

Lymphocyte transformation. Six milliliters of blood was mixed with 4 ml of a solution containing 6% dextran and 250 U of heparin. After sedimentation of erythrocytes, the leukocyte-rich plasma was aspirated; the cells were washed three times in minimum essential medium, suspended in minimum essential medium with 20% fetal calf serum, 1 mM L-glutamine, and 100 μg of streptomycin per ml, and counted. One-milliliter portions of the final suspension containing 5×10^5 mononuclear cells were added to glass tissue culture tubes. PHA-M (Difco Laboratories, Detroit, Mich.) was diluted in 5 ml of phosphate-buffered saline, and 10-μl portions of various dilutions (undiluted, 1:2, and 1:20) were added to triplicate sets of cultures. picHSA was added to triplicate sets of cultures in amounts varying from 5 to 50 μg/ml. Control cultures contained no mitogen or antigen. After incubation for 3 days (PHA) or 6 days (picHSA), cultures were pulse-labeled with [*methyl*-^{3}H]thymidine and harvested by methods previously described (12). Results were expressed as mean disintegrations per minute for each triplicate set of cultures.

T-lymphocyte rosette formation. Three milliliters of heparin-treated guinea pig blood was diluted three-fold with 0.9% saline, layered over a Ficoll-Isopaque gradient, and spun at $400 \times g$ for 40 min (2). The interface, which was greater than 90% lymphocytes, was aspirated, and the cells were washed in Hanks basic salt solution. Lymphocytes (2×10^5 in 0.1 ml), washed rabbit erythrocytes (10^7 in 0.1 ml), and one drop of washed latex particles were incubated together for 10 min at 37°C, centrifuged at $150 \times g$ for 5 min, and placed on ice overnight. After gentle resuspension, 400 lymphocytes were counted, and lymphocytes with three or more erythrocytes attached were counted as rosettes (8, 23).

Skin tests. Guinea pigs were preimmunized with picHSA as described above. Skin tests were performed 7 days before inoculation with influenza and were repeated 2 days after viral inoculation. Animals were injected intradermally on the shaved flank with 10 μg of picHSA in 0.1 ml of saline. Two perpendicular

diameters of the reaction site were measured and averaged at 24 h, the time of maximum skin test response.

RESULTS

All 18 ferrets inoculated with influenza A, Hong Kong, experienced a rise in body temperature (mean elevation, 2.6°C), which was maximal on day 3 after inoculation; 8 had coryza and appeared lethargic. The mean duration of illness was 4.5 days, and all indicators of infection had returned to base line by day 6. None of the control animals showed any of the above signs. Influenza virus was isolated from the nasal washes of 16 of the 18 animals on day 3. All infected animals (but no controls) showed a significant rise in hemagglutination inhibition antibody titer to influenza (preinoculation geometric mean titer, <1:8; postinoculation, 1:1,288). One of two ferrets had virus isolated from lung tissue in low titer (<1 log). Pathological examination of lung tissue revealed minimal peribronchiolar mononuclear cell proliferation but no areas of pneumonitis.

Infection with influenza A, Hong Kong, did not diminish the response to PHA (Fig. 1a). When the maximum responses for each animal before inoculation, during illness, and after convalescence were compared, no significant differences were found ($P > 0.5$, Student's t test for paired samples). Thymidine incorporation in the cultures that contained no mitogen was unchanged during infection (636 dpm) when compared with preinoculation values (717 dpm) and convalescent values (644 dpm). Comparison of infected with control groups on each of the 3 days studied revealed no significant differences between the two groups ($P > 0.1$ for each day, Wilcoxan rank sum test). Evaluation of the response to suboptimal doses of PHA, rather than the maximum response, again showed no differences between control and infected groups.

The six ferrets inoculated with influenza A, PR8, showed illness indistinguishable from influenza A, Hong Kong, infection; all had coryza and temperature elevations of from 2.0 to 2.9°C on day 3 after inoculation. No control animals became ill. Influenza virus was isolated from nasal washes in all six infected ferrets. Virus was not grown in cultures taken from the lung tissue of two ferrets. Pathological examination of these lungs revealed more extensive accumulation of mononuclear cells in the peribronchiolar tissue than in animals infected with influenza A, Hong Kong; however, pneumonitis was not present.

During the height of the illness on day 3, PHA responsiveness was not diminished in comparison with preinoculation and convalescent values (Fig. 1b). No significant differences were discerned between control groups and infected groups on each of the 3 days studied. Cultures containing no mitogen showed no significant changes in thymidine uptake during the course of the infection (1,627 dpm on day −7, 1,759 dpm on day 3, and 3,377 dpm on day 15).

On days 2 and 3 after inoculation with influenza A, Hong Kong, 23 of 24 guinea pigs shed influenza virus from the anterior nares. No signs of overt infection developed in any of the animals—temperatures remained normal, and lethargy and coryza were absent.

There were no significant differences between the infected and control groups in the maximum PHA stimulation on any of the days studied ($P > 0.1$ for each day, Wilcoxan rank sum test; Fig. 2a). When the postinoculation PHA response of each guinea pig was compared with its base-line

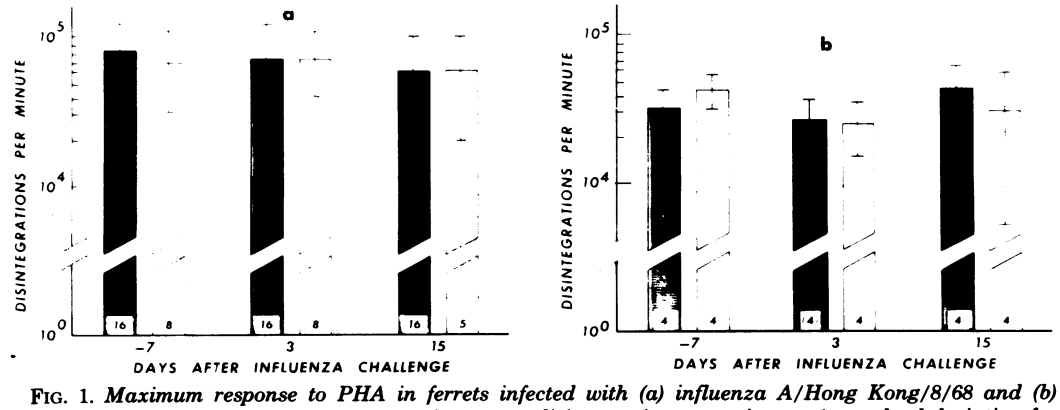

FIG. 1. *Maximum response to PHA in ferrets infected with (a) influenza A/Hong Kong/8/68 and (b) influenza A/PR/8/34. Results are expressed as mean disintegrations per minute ±1 standard deviation for infected (■) and control (□) ferrets on each of the days studied. Numbers within each box indicate the number of animals studied.*

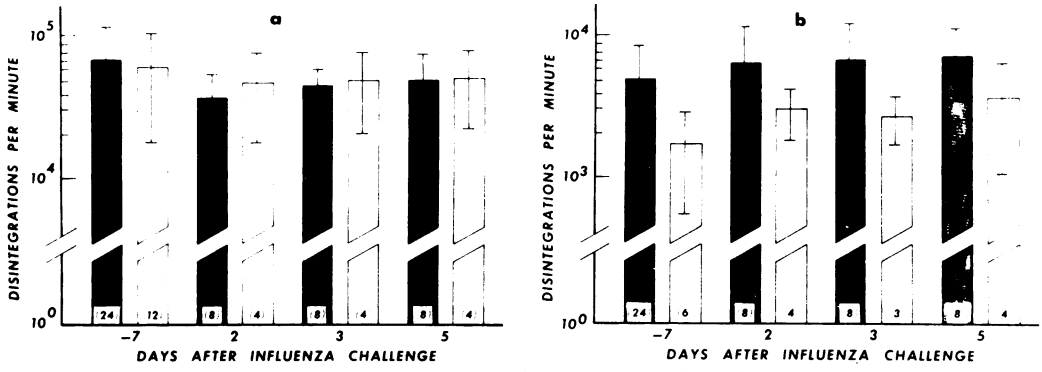

FIG. 2. *(a) Maximum response to PHA in guinea pigs inoculated with influenza A/Hong Kong/8/68. (b) Maximum response to picHSA in guinea pigs inoculated with influenza A/Hong Kong/8/68. Results are expressed as mean disintegrations per minute ± 1 standard deviation for infected (■) and control (○) guinea pigs on each of the days studied. Numbers within each box indicate the number of animals studied.*

response, a decrease was noted on day 2 ($P < 0.05$, Student's t test for paired samples) but not on days 3 or 5. Control animals also showed a diminished stimulation on day 2 as compared with base-line values; however, this change was not statistically significant. When the response to a suboptimal dose of PHA, rather than the maximum response, was evaluated, no differences were found between control and infected animals.

Lymphocyte transformation to picHSA was unchanged in control and infected guinea pigs throughout the observation period (Fig. 2b). Control animals consistently showed less deoxyribonucleic acid synthesis in response to picHSA than those in the experimental group; the reason for this is unclear.

The percentage of circulating T lymphocytes was similar for control and infected animals in the initial base-line study and did not change significantly for either group during the postinoculation period (Fig. 3).

Twenty-four-hour skin test reactions to picHSA on day 2 after influenza A, Hong Kong, inoculation were positive in all seven control animals and in all eight infected animals (Fig. 4).

Passive transfer of delayed hypersensitivity to picHSA was accomplished in all recipient strain 13 guinea pigs. All animals were shedding virus at the time of skin testing. The average diameter of the transfer reaction was 6.3 mm; five of the eight animals had reactions greater than 5 mm in diameter with a 2+ induration. This is within the range of skin test responsiveness found in previous experiments with noninfected animals that received sensitized cells.

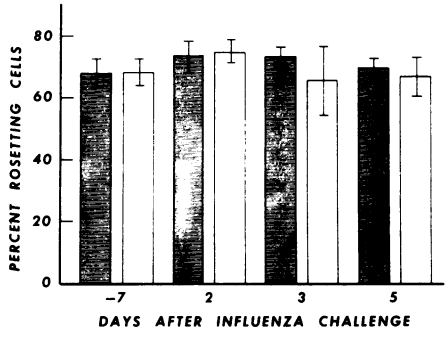

FIG. 3. *Percentage of T lymphocytes in guinea pigs inoculated with influenza A/Hong Kong/8/68. Results are shown as mean percentage ± standard deviation for infected (■) and control (□) guinea pigs on each of the days studied.*

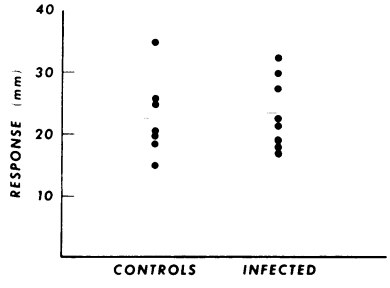

FIG. 4. *Skin test response to picHSA in infected and control guinea pigs on day 2 of influenza A/Hong Kong/8/68 infection.*

DISCUSSION

These studies were undertaken to determine the effect of localized respiratory tract viral infection on systemic cell-mediated immunity. Previous studies in humans raised the possibility that mild upper respiratory tract infections did not lead to immunosuppression, although severe pneumonitis had a significant immunosuppressive effect (10, 11).

Influenza infection in ferrets has been extensively studied (6, 7, 13, 17, 22, 27). The pathological changes are most prominent in the upper airways; desquamation of nasal epithelium and infiltration of the submucosa of the nose with inflammatory cells typify the histological reaction (13). However, with the use of certain pneumotropic strains (21, 26) or other strains that have been serially passaged in ferrets (19), pneumonitis can be produced in these animals. The Hong Kong strain of influenza used in the present study had been passaged in eggs and had not produced pneumonitis in other studies (17). None of the animals had more than mild to moderate clinical illness (coryza, lethargy, fever), and pathological examination of the lungs revealed only mild peribronchiolar accumulations of mononuclear cells.

During illness, no suppression of lymphocyte response to PHA was observed. This lack of systemic immunosuppression was confirmed with a second strain of influenza A, the PR8 strain. Increased incorporation of thymidine by unstimulated lymphocytes, as described by Buckley et al. during influenza pneumonitis in humans (3), was not observed in the ferret lymphocyte cultures. Subtle differences in PHA stimulation, such as diminished response to suboptimal doses of the mitogen, that have been described in a study of measles (5) were not found during influenza in the ferrets.

Previous studies had indicated that skin test reactions were difficult to elicit in the ferret (18; C. Kauffman, unpublished data); therefore, skin tests were not performed. Spontaneous T-cell rosette formation was extensively sought but not found in studies with erythrocytes from 15 different species.

Influenza infection in guinea pigs was studied because it serves as a good model of localized upper respiratory tract infection and because multiple parameters of cell-mediated immunity can be investigated in these animals. Viral multiplication is restricted to the nasal turbinates, with minimal changes in the nasal mucosa (25); thus, this model represents an even more localized upper respiratory tract infection than that seen in ferrets. Virus that has been serially passaged in guinea pigs does not produce pneumonitis (25; Phair et al., submitted for publication).

A depression of PHA-induced lymphocyte deoxyribonucleic acid synthesis was found on day 2 after influenza inoculation. This was evident only when the PHA response of each animal was compared with its base-line response, but was not apparent when infected animals were compared with control animals on day 2. It is difficult to relate the decreased mitogenic response on day 2 to the viral infection because the control animals showed a similar fall in their response to PHA. Both skin tests and lymphocyte transformation to picHSA were intact on day 2. Significant immunosuppression, if present, should have been reflected in these indicators as well as in the PHA response. The percentage of circulating T lymphocytes did not change in guinea pigs after viral challenge. Transfer of sensitized spleen cells resulted in positive skin tests in the recipients, although all were shedding virus from the anterior nares at the time of skin testing. Influenza had no effect on either the recruited recipient mononuclear cells or the sensitized effector donor lymphocytes, contributing to the delayed hypersensitivity skin test response.

These studies indicate that mild influenza infection in ferrets and guinea pigs is not associated with significant suppression of systemic cell-mediated immunity, thus confirming the results of previous work in humans (11). Whether the converse—that influenza pneumonitis produces immunosuppression—can be shown in animals will have to be determined with a strain of virus that consistently produces severe pneumonitis.

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