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Distemper virus infection in ferrets: an animal model of measles-induced immunosuppression

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SUMMARY

Distemper virus is very similar antigenically to measles virus, and the disease produced in ferrets by distemper is a systemic illness quite similar to measles infection in humans. Using an attenuated strain of distemper virus, we produced a mild systemic illness in ferrets and were able to study the effects of the viral infection on cell-mediated immunity (CMI). Beginning on day 5 after viral inoculation and continuing to day 30, infected ferrets showed a marked lymphopenia, with a reduction in total numbers of all lymphocyte subpopulations studied. Transformation of circulating lymphocytes to the mitogens phytohemagglutinin, concanavalin A, and pokeweed mitogen was suppressed on day 5, reached a nadir by days 8 to 11, and returned toward normal by days 23 to 30 after viral inoculation. Production of macrophage migration inhibitory factor by splenic macrophages was diminished during distemper infection. In contrast to marked suppression of these *in vitro* assays for CMI, delayed hypersensitivity skin test responses were only slightly diminished in animals infected with distemper virus. This model should prove useful in exploring the mechanisms of measles induced immunosuppression.

INTRODUCTION

Distemper virus infection of dogs and ferrets bears great resemblance to measles virus infection in humans. Antigenically, distemper virus is very similar to measles virus, and, with the exception of the rash, the illness produced by distemper in dogs and ferrets is strikingly similar to that seen with measles in humans (Appel & Gillespie, 1972; Imagawa, 1968). Fever, prostration, coryza, conjuctivitis and encephalitis are found in both infections, although the latter is far more often found with distemper than with measles (Imagawa, 1968).

That measles leads to skin test anergy has been known since 1908 (von Pirquet, 1908); it is now clear that measles infection in humans also diminishes *in vitro* lymphocyte mitogenic proliferation (Finkel & Dent, 1973; Wesley, Coovadia & Henderson, 1978); may lead to the appearance of serum suppressive factors (Whittle *et al.*, 1978); decreases T lymphocyte numbers (Wesley *et al.*, 1978; Whittle *et al.*, 1978); and inhibits lymphokine production (Joffe & Rabson, 1978). In puppies, distemper infection has been shown to have effects on the cellular immune system similar to those seen in humans with measles (Krakowka, Cockerell & Koestner, 1975; Mangi *et al.*, 1975). Using an attenuated strain of distemper virus, we produced mild distemper virus infection in ferrets, mammals well suited to serial studies of cell-mediated immunity (CMI) (Kauffman, 1981). This report describes the depression of CMI observed during distemper virus infection in ferrets.

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MATERIALS AND METHODS

Animals. Male ferrets, age 3–6 months (Marshall Research Animals, North Rose, New York) were housed in rabbit cages and fed Purina cat chow and water *ad libitum*. Animals infected with distemper virus were housed in a separate room from control animals. Blood was obtained by cardiac puncture after inducing anaesthesia with ketamine hydrochloride (22 mg/kg given subcutaneously). Spleens were removed after deep phenobarbital anaesthesia and exsanguination.

Hartley strain guinea-pigs (Camm Research Institute, Wayne, New Jersey) were used to obtain peritoneal macrophages for macrophage migration inhibitory factor (MIF) studies.

Immunization. All animals were immunized in the axillary skin fold with 0·5 ml of a suspension of 800 units streptokinase (SK) (Lederle Inc., Pearl River, New York) in Freund's complete adjuvant (DIFCO, Detroit, Michigan) on two separate occasions, 12 days apart.

Distemper infection. Experimental animals were inoculated intramuscularly with 2 ml of a suspension of distemper virus grown in primary canine kidney tissue culture cells (10^4 $TCID_{50}$/ml) (Tissuevax) (Pitman-Moore Inc., Washington Crossing, New Jersey). Observations were made twice daily regarding lethargy, food intake, and rectal temperature.

Experimental design. Twenty-four days after immunization with SK was initiated, studies of delayed hypersensitivity skin test reactions, MIF production, and lymphocyte subpopulations were begun on 30 ferrets (15 inoculated with distemper virus and 15 controls). Baseline skin tests were performed on all animals; skin tests were repeated in groups of 10 animals on days 4, 11, and 18 following distemper virus inoculation. These same animals were sacrificed following reading of the 48 hr skin test, and lymphocyte subpopulation and MIF studies were performed.

A separate group of 24 ferrets (12 inoculated with distemper virus and 12 controls) were utilized for serial studies of lymphocyte proliferation beginning 24 days after immunization with SK was initiated. Studies were obtained on day 0 and on days 3, 5, 8, 11, 16, 23 and 30 following virus inoculation.

Lymphocyte subpopulations. Peripheral white blood cell and differential counts were done by standard methods. Lymphocytes were obtained by Ficoll-Isopaque separation of peripheral blood lymphocytes (Böyum, 1968). Cells were washed three times in Hanks' basic salt solution (HBSS) and resuspended at a concentration of 4×10^6 cells/ml in HBSS.

Complement receptor bearing lymphocytes were identified by their ability to form rosettes with zymosan particles (Sigma Chemical Co., St Louis, Missouri) coated with C3 from fresh human serum (Kauffman & Bergman, 1981; Mendes, Miki & Peikinho, 1974). Mononuclear cells (0·2 ml) were mixed with 0·4 ml zymosan particles at 37°C for 15 min, centrifuged at 150 ***g*** for 5 min, and placed at 4°C for 2 hr. Among 400 lymphocytes counted, those cells with three or more zymosan particles attached were recorded as lymphocytes bearing complement receptors.

FC receptor bearing lymphocytes were identified by their ability to form rosettes with sheep erythrocytes coated with rabbit IgG anti-sheep erythrocyte antibodies (Cordis Laboratories, Miami, Florida) (Kauffman & Bergman, 1981; Clements & Levy, 1978). Sensitized erythrocytes (0·2 ml) were mixed with 0·2 ml of ferret mononuclear cells at 37°C for 15 min, centrifuged at 150 ***g*** for 5 min, and placed at 4°C for 2 hr. Among 400 lymphocytes counted, those with three or more rosettes attached were recorded as Fc receptor bearing lymphocytes.

Immunoglobulin was detected on the surface of lymphocytes using an indirect fluorescent antibody technique described previously (Kauffman & Bergman, 1981). Lymphocytes were treated in the cold with rabbit anti-ferret immunoglobulin, washed, and treated with goat anti-rabbit immunoglobulin conjugated with fluorescein (Meloy Laboratories, Springfield, Virginia). Four hundred lymphocytes were read for surface fluorescence using a Zeiss standard microscope with epi-fluorescence excitation.

Skin tests. Delayed hypersensitivity skin test reactions to preservative free purified protein derivative (PPD) (Parke-Davis Inc., Detroit, Missouri) were performed as described previously (Kauffman, 1981), using 200 μg PPD in 0·1 ml 0·9% NaCl. At 24, 32, and 48 hr, induration of the skin test site and adjacent uninoculated skin was measured with a vernier calipers, and the difference in thickness was recorded in mm.

Lymphocyte transformation. Peripheral blood mononuclear cells obtained by Ficoll-Isopaque gradient separation (Böyum, 1968), were washed three times in RPMI 1640 (GIBCO, Grand Island, New York), counted, and suspended at a concentration of 25×10^5 mononuclear cells/ml in RPMI 1640 with 10% heat-inactivated fetal calf serum (Flow Laboratories, Cockeysville, Maryland), 2mM L-glutamine, and 100 μg streptomycin/ml. The cell (0·2 ml) suspension was added to each well of a flat bottomed microtitre plate (Linbro Inc., Hamden, Connecticut). The following mitogens diluted in RPMI 1640 were added in a 0·01 ml volume to triplicate sets of cultures: concanvalin A (con A) (Pharmacia Inc. Piscataway, New Jersey), 0·5 μg, 2·5 μg, and 12·5 μg; phytohemagglutinin-M (PHA) (DIFCO), 1 : 30, 1 : 10, and undiluted; and pokeweed mitogen (PWM) (GIBCO), 1 : 30, 1 : 10, and undiluted. Control cultures received 0·01 ml of RPMI 1640.

After incubation at 37°C in a humidified 5% CO_2 atmosphere for 3 days, cultures were pulse-labelled for 4 hr with 1 μCi tritiated thymidine (specific activity 6 Ci/mmole) (Schwarzmann Inc., Orangeburg, New York) then harvested using a multiple automated sample harvester (Microbiological Associates, Rockville, Maryland) (Kauffman, Schiff & Phair, 1978). Isotope incorporation was measured in a liquid scintillation counter. The dilution of mitogen giving the maximum response was used for analysis; results were expressed as the mean counts per minutes (c.p.m.) for each triplicate set of cultures.

MIF assay. Splenic lymphocytes were obtained as previously described (Kauffman, 1981), and the mononuclear cells were purified on a Ficoll-Isopaque gradient (Böyum, 1968). Cultures were prepared in 1 ml plastic tubes with loose fitting caps using 2 ml of a suspension of 1×10^7 mononuclear cells/ml in RPMI 1640 with 2 mM L-glutamine and 100 μg streptomycin/ml. No serum was added to these cultures. SK, which had been dialyzed 48 hr against 0·9% NaCl, was added to stimulated cultures at a concentration of 160 units/ml; control cultures contained no antigen. After 24 and 48 hr incubation, supernatants were removed and combined. Antigen was added to the control supernatants and the material was dialyzed against distilled water for 24 hr, lyophilized, and stored at −70°C. The lyophilized supernatants were reconstituted to a volume equal to the original volume with RPMI 1640 with 10% heat-inactivated guinea-pig serum added.

Peritoneal macrophages obtained from Hartley strain guinea-pigs were processed as described previously (Kauffman, 1981). The cells were suspended in RPMI 1640 with 10% heat-inactivated guinea-pig serum and placed into capillary tubes, which were placed in 16 mm wells of a 24 well tissue culture plate (Linbro Inc.). One millilitre of reconstituted MIF-containing or control supernatant was added to each well. All assays were run in triplicate, and the results were averaged. Migration was measured with a projecting microscope after 24 hr incubation at 37°C in a humidified 5% CO_2 atmosphere. The area of migration was calculated with a planimeter and the percentage migration inhibition calculated by the following formula:

$$\% \text{ inhibition} = 1 - \frac{\text{migration in well with supernatant from stimulated culture}}{\text{migration in well with supernatant from control culture}} \times 100$$

Greater than 20% inhibition of migration was considered positive.

Statistical methods. Data were analysed using Student's *t*-test.

RESULTS

Illness

Ferrets inoculated with distemper virus developed lethargy, anorexia, and a rise in rectal temperature of 1·0–1·5°F beginning on the 4th day after inoculation and lasting 4 days. The animals were lethargic for 3–4 days, but none appeared seriously ill. No animals manifested central nervous system signs. Seven animals manifested a recrudescence of illness with lethargy, anorexia, hypothermia and ruffled coat on days 22 to 25 following distemper inoculation. Four of these seven animals became tachypneic and appeared severely ill; three of the four died with extensive pneumonitis due to gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). In these three animals, pathologic examination of lungs, spleen, and liver did not reveal giant cell formation or inclusion bodies suggesting active distemper infection.

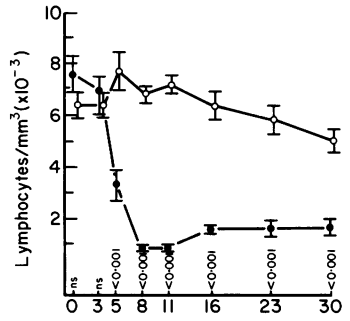

Fig. 1. Peripheral blood lymphocyte counts expressed as mean number of lymphocytes ± s.e.m., in ferrets with distemper. (● = experimental; ○ = control).

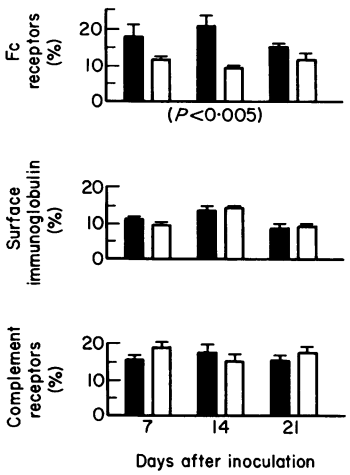

Fig. 2. Peripheral blood lymphocyte subpopulations expressed as mean percentage of lymphocytes ± s.e.m. in ferrets with distemper infection. (■ = experimental; □ = control).

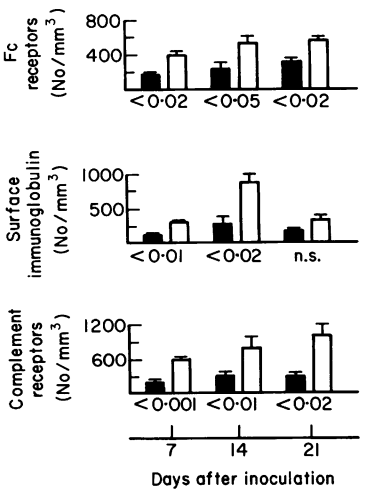

Fig. 3. Peripheral blood lymphocyte subpopulations, expressed as mean number of lymphocytes ± s.e.m., in ferrets with distemper infection. (■ = experimental; □ = control).

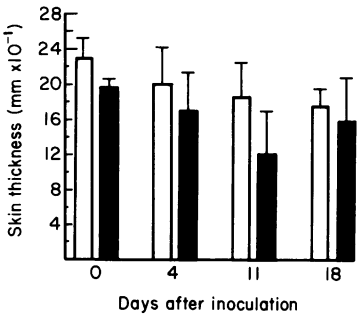

Fig. 4. Delayed hypersensitivity skin test reactions to PPD in ferrets with distemper. Data are expressed as the increase in thickness of skin test site compared with uninoculated skin in mm $\times 10^{-1}$ ($\bar{x} \pm$ s.e.m.). (■ = experimental; □ = control).

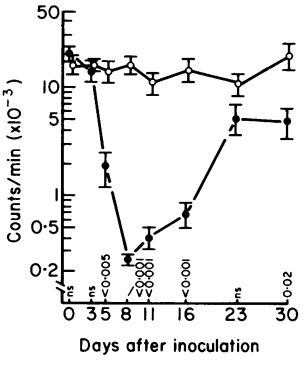

Fig. 5. Maximum response of peripheral blood lymphocytes to stimulation with phytohaemagglutinin (PHA) in ferrets with distemper infection. The data are expressed as mean counts per minute ± s.e.m. (● = experimental; ○ = control).

Lymphocyte subpopulations

Animals given distemper virus showed marked lymphopenia beginning on day 5 and continuing through the end of the experiment on day 30 (Fig. 1). Enumeration of lymphocyte subpopulations revealed that the relative percentages of cells with surface immunoglobulin and receptors for complement remained unchanged from those shown by controls, while the percentage of circulating cells bearing Fc receptors increased (Fig. 2). However, due to the marked lymphopenia, all subpopulations decreased when expressed in terms of total numbers of cells (Fig. 3).

Delayed hypersensitivity skin tests

Skin tests to PPD were decreased on day 11 after distemper inoculation, but the differences between experimental and control ferrets were not statistically significant (Fig. 4).

Lymphocyte transformation

Ferrets with distemper manifested a markedly diminished response to the mitogens PHA, con A, and PWM, beginning on day 5, reaching a maximum on days 8 to 11, and returning toward levels shown by control animals by days 23–30 (Figs. 5–7). The same significant depression was noted if the data were expressed as mean stimulation index, which is c.p.m. stimulated cultures/c.p.m. unstimulated cultures rather than mean c.p.m. All animals which had a recrudescence of illness had markedly abnormal responses to mitogenic stimulation from day 5 until day 16 when they were last studied.

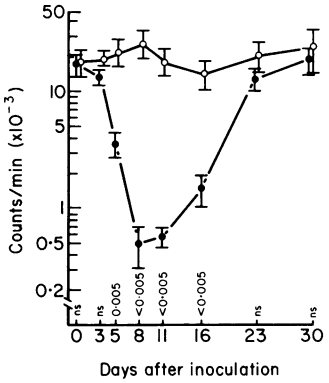

Fig. 6. Maximum response of peripheral blood lymphocytes to stimulation with concanavalin A (con A) in ferrets with distemper infection. The data are expressed as mean counts per minute ± s.e.m. (● = experimental; ○ = control).

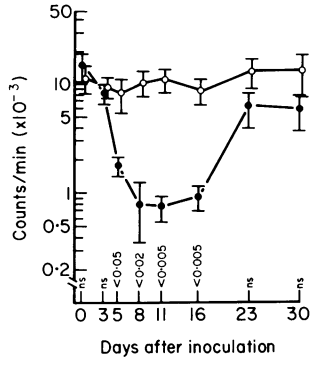

Fig. 7. Maximum response of peripheral blood lymphocytes to stimulation with pokeweed mitogen (PWM) in ferrets with distemper infection. The data are expressed as mean counts per minute ± s.e.m. (● = experimental; ○ = control).

Table 1. MIF production by splenic lymphocytes of ferrets infected with distemper virus

Days after inoculation	Percentage migration inhibition ($\bar{x} \pm$ s.e.m.) Distemper infected	Controls	*P*
7	7·2 ± 3·2 (5)	30·6 ± 8·6 (5)	< 0·05
14	13·8 ± 3·8 (5)	31·0 ± 5·8 (5)	< 0·05
21	12·8 ± 9·1 (5)	24·6 ± 17·0 (5)	n.s.

Number of ferrets in each group are given in parentheses

> 20% inhibition is considered positive.

MIF assay

Ferrets infected with distemper virus showed a diminished capacity to produce MIF (Table 1). This effect was more marked on days 7 and 14 after inoculation; two of five infected ferrets produced MIF when studied on day 21.

DISCUSSION

Depression of CMI occurs during the course of many different viral infections in humans (Kantor, 1975; Notkins, Merganhagen & Howard, 1970). The proclivity of measles virus to depress CMI has been amply documented (von Pirquet, 1908; Finkel & Dent, 1973; Wesley *et al.*, 1978; Whittle *et al.*, 1978; Joffe & Rabson, 1978; Desai, Strashoon & Wesley, 1978, Fireman, Friday & Kumate, 1969; Hughes, Smith & Kim, 1968; Ilonen *et al.*, 1980). Delayed hypersensitivity skin test responses were noted to be diminished in the presence of the measles exanthem more than 70 years ago (von Pirquet, 1908); skin test anergy has been confirmed recently in children with active measles infection (Wesley *et al.*, 1978; Desai *et al.*, 1978) as well as in those vaccinated with an attenuated strain of measles virus (Fireman *et al.*, 1969, Hughes *et al.*, 1968).

In vitro assays of CMI have shown measles induced depression of lymphocyte function, but different investigators have noted varying degrees of immunosuppression. For example, Wesley *et al.*, (1978) showed diminished mitogen responsiveness during acute measles infection, while Joffe & Rabson (1978) found normal mitogenic responsiveness. Several groups have noted diminished responses only with suboptimal doses of PHA (Finkel & Dent, 1973; Whittle *et al.*, 1978), and others have found serum inhibitors to lymphocyte transformation (Whittle *et al.*, 1978). It is unclear why such differences have been noted in these studies, but certainly the severity of disease, the nutritional status of the children, and the laboratory techniques were not controlled between these studies. In contrast to mitogenic responsiveness, antigenic stimulation appears to be consistently depressed in those with measles infection and in those vaccinated with an attenuated strain of measles (Whittle *et al.*, 1978; Fireman *et al.*, 1969; Ilonen *et al.*, 1980).

Lymphokine production has been studied infrequently in children with measles; both direct assays for LIF and indirect assays for MIF and LIF have been abnormal in those studied (Whittle *et al.*, 1978; Joffe & Rabson, 1978; Desai *et al.*, 1978).

Mechanisms for the above mentioned measles-associated immunodepression have not been completely elucidated although several studies have attempted to look at the interaction of measles virus with different mononuclear cell populations. Several different groups have now shown that measles virus can replicate in mitogen stimulated T and B lymphocyte populations, T and B lymphoblastoid cell lines and, albeit less well, in monocytes (Gallagher & Flanagan, 1976; Joseph, Lampert & Oldstone, 1975; Sullivan *et al.*, 1975). In children with acute measles infection, circulating lymphocytes containing measles virus are readily lysed on incubation with autologous serum and complement (Whittle *et al.*, 1978). These same children have decreased circulating T cell numbers.

A relevant animal model would allow further exploration of the mechanisms involved in measles induced suppression of CMI. Although monkeys can be infected with measles virus, this is not an easily studied animal species (Hicks, Sullivan & Albrecht, 1977). Measles virus can be adapted to mice, but this model does not replicate the systemic illness of humans (McFarland, 1974; Neighbour, Rager-Zisman & Bloom, 1978). Similar to measles in humans, distemper in ferrets and dogs is a systemic illness with viremia, fever, and multi-organ involvement (Appel & Gillespie, 1972; Imagawa, 1968). The distemper virus is so closely related to measles virus that immunization with measles can protect dogs and ferrets from distemper infection (Imagawa, 1968).

Our studies with ferrets showed results similar to those obtained in gnotobiotic dogs infected with distemper (Krakowka *et al.*, 1975; Mangi *et al.*, 1975; McCullough, Krakowka & Koestner, 1974). Marked lymphopenia was prominent throughout the course of the infection. All lymphocyte subpopulations showed a striking decrease in total numbers because of the lymphopenia, although there was a relative rise in the percentage of circulating Fc receptor bearing cells. Since no marker for ferret T lymphocytes has yet been identified (Kauffman & Bergman, 1981), it is not known if a depression of T cell numbers occurs as has been found in children with measles (Wesley *et al.*, 1978; Whittle, *et al.*, 1978).

In addition, to marked lymphopenia, the *in vitro* function of both circulating and splenic lymphocytes was depressed in distemper infected ferrets. In contrast to some studies in children (Joffe & Rabson, 1978), but in concert with others (Wesley *et al.*, 1978), the mitogenic response was

markedly suppressed during active infection. This depression occurred in cultures incubated in fetal calf serum, inferring an intrinsic cellular defect rather than serum related immunosuppression (Whittle *et al.*, 1978). MIF production by splenic lymphocytes was absent on days 7 and 14 but appeared to be returning to normal by day 21.

In contrast to the *in vitro* studies showing diminished lymphocyte function, delayed hypersensitivity skin test responses were only slightly diminished in ferrets with distemper. A similar dichotomy between *in vivo* and *in vitro* assays for CMI was noted by Krakowka *et al.*, who noticed no enhanced allograft survival but markedly diminished *in vitro* mitogenic responsiveness in gnotobiotic puppies with distemper (Krakowka *et al.*, 1975).

The attenuated strain of distemper virus which we used caused a mild infection without encephalitis and mortality from distemper, thus making it more likely that the immunosuppression observed was viral induced and not related to the non-specific immunosuppression seen with overwhelming illness (Kauffman *et al.*, 1976). Virulent strains of distemper usually cause a severe acute illness with a high fatality rate in ferrets and, thus, would not be suitable for serial studies of CMI (Dunkin & Laidlaw, 1926). Although we did not see progressive distemper with the attenuated virus we used, three ferrets did die of pneumonitis. It is likely that the pneumonia was secondary to the immunosuppression induced by the viral infection. We did not assess the effect of distemper infection on neutrophil function. It is possible that a viral induced defect in neutrophil function could have contributed to the development of gram-negative bacillary pneumonia in these three ferrets.

Further studies with distemper infection in ferrets should lead to an elucidation of the mechanisms of the immunosuppression we have observed.

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