DEVELOPMENTAL AND COMPARATIVE IMMUNOLOGY, Vol. 5, pp. 125-134, 1981
0145-305X/81/010125-10$02.00/0 Printed in the USA.
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CELL-MEDIATED IMMUNITY IN FERRETS DELAYED DERMAL HYPERSENSITIVITY, LYMPHOCYTE TRANSFORMATION, AND MACROPHAGE MIGRATION INHIBITORY FACTOR PRODUCTION

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ABSTRACT. Cell-mediated immune reactions - delayed dermal hypersensitivity, lymphocyte transformation, and macrophage migration inhibitory factor production - were investigated in the ferret. Ferrets immunized with streptokinase in complete Freund's adjuvant had skin test responses characterized by induration with little erythema; skin test biopsies showed mononuclear cell infiltration. In vitro transformation of peripheral blood or splenic lymphocytes was elicited by phytohemagglutinin, concanavalin A, pokeweed mitogen, and streptokinase; macrophage migration inhibitory factor was produced by spleen cells from immunized ferrets. These studies confirm the usefulness of certain tests of cell-mediated immunity in ferrets.

INTRODUCTION

The ferret, Mustela furo, a mammal closely related to weasels, minks, and skunks, is used frequently as an animal model for human viral infections. For example, the pathogenesis of influenza A infection in the ferret is almost identical to that in humans (1), respiratory syncytial virus infection in young ferrets is similar to that noted in human infants (2), and distempter virus causes a systemic illness in ferrets similar to that which occurs in children with measles (3). As a consequence of numerous viral studies performed in ferrets, much is known about their humoral immune system (4), including detailed information on the ontogeny of humoral immunity (5). According to recent studies, cell-mediated immunity (CMI) may be as important as humoral immunity in the pathogenesis of some viral infections (6,7), but data on cell-mediated immune reactions in ferrets and closely related mammals, such as minks, are meager (8-11). The purpose of these studies was to investigate various aspects of CMI - delayed hypersensitivity skin tests (DTH), lymphocyte transformation (LT), and macrophage migration inhibitory factor (MIF) production - in the ferret as a prelude to further studies on CMI during viral infections.

METHODS

Ferrets.

Ferrets of either sex, age three to twelve months (Marshall Research Animals, North Rose, NY) were housed in rabbit cages and fed Purina cat chow and water ad libitum. Blood was obtained by cardiac puncture after inducing anesthesia with 22 mg ketamine hydrochloride per kg given subcutaneously. Spleen were removed after deep phenobarbital anesthesia and exsanguination.

Guinea pigs.

Hartley strain guinea pigs (Camm Research Institute, Wayne, NJ) were used to obtain macrophages for MIF studies.

Immunization.

Ferrets were immunized twice, twelve days apart, with 500 units streptokinase (SK) (Lederle, Inc., Pearl River, NY) in complete Freund's adjuvant (Difco, Inc., Detroit, MI) (0.5 ml) in the axillary skin fold.

Skin tests.

Skin tests were performed in 22 ferrets by injecting 200 μg (10,000 tuberculin units) preservative-free purified protein derivative (PPD) (Parke-Davis, Inc., Detroit, MI) in 0.1 ml 0.9% saline or 400 units SK in 0.1 ml 0.9% saline intradermally into the shaved flank. The diameter of erythema was measured and the induration of skin-tested and adjacent normal skin was determined with a vernier calipers at 0, 4, 24, 32, 40, 48, and 72 hours. Data were expressed as the difference in skin fold thickness in mm between skin-tested and normal skin. Dermal biopsies were performed on five ferrets at 30 and 48 hours.

Cell separation.

Blood obtained by cardiac puncture was diluted three-fold in heparinized saline (0.9%) and layered over a ficoll-isopaque gradient to separate mononuclear cells (12). Lymphocytes constituted more than 90% of the cells in the upper layer. Spleen cells were harvested by repeatedly injecting RPMI-1640 medium (GIBCO, Inc., Grand Island, NY) into the aseptically removed spleens using a 22 gauge needle and a 3 ml syringe. The spleen cell preparation was diluted three-fold and placed on a ficoll-isopaque gradient for separation of mononuclear cells (12). Lymphocytes then comprised more than 85% of the cells in the upper layer.

Lymphocyte transformation.

Mononuclear cells obtained from peripheral blood or spleens were washed three times in RPMI-1640, counted, and suspended at a concentration of 25 x 10^5 cells/ml in RPMI-1640 with 10% heat inactivated serum, 2mM l-glutamine, and 100 μg streptomycin per ml. Ferret, human, and fetal calf serum were tested. Two-tenths ml of cell suspension was added to each well of a flat-bottomed microtiter plate (Linbro, Inc. Hamden, CT). The following mitogens and antigens were added in 0.01 ml volumes to triplicate sets of cultures: concanavalin A (CON A) (Pharmacia, Inc., Piscataway, NJ), from 1 μg to 12.5 μg

per culture; phytohemagglutinin-M (PHA) (Difco, Inc.), from a 1:30 dilution to an undiluted solution per culture; pokeweed mitogen (PWM) (GIBCO, Inc.), from a 1:50 dilution to an undiluted solution per culture; SK, 4 units to 80 units SK per culture (after the SK had been dialyzed against 0.9% saline for 48 hours). All mitogens and antigens were diluted in RPMI-1640. Control cultures contained neither mitogen nor antigen.

After incubation at 37°C in a 5% CO_2, 95% air, humidified atmosphere for periods varying from 2 to 8 days, cultures were pulse-labelled 4 hours before harvest with 1 µCi tritiated thymidine (specific activity 6 Ci/mmole) (Schwarzmann, Inc., Orangeburg, NY). Cultures were harvested using a multiple automated sample harvester (MASH) (Microbiological Associates, Rockville, MD) and isotope incorporation measured in a liquid scintillation counter. Results were expressed as the mean counts per minutes (CPM) for each triplicate set of cultures.

MIF assay.

Mononuclear cells obtained from peripheral blood or spleens were prepared as described above. Cultures were prepared in 1 ml plastic tubes with loose-fitting caps using 2 ml of a suspension of 1 x 10^7 mononuclear cells/ml for spleen cells and 5 x 10^6 cells/ml for peripheral blood cells in RPMI-1640 with 2 mM L-glutamine and 100 µg streptomycin per ml. No serum was added to these cultures. SK, 160 units/ml, was added to stimulated cultures; control cultures contained no antigen. The supernatants were removed after 24 and 48 hours incubation and combined. Antigen was added to the control supernatants; the material was dialyzed against distilled water for 24 hours, 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.

Macrophages were washed from the peritoneal cavity of normal Hartley strain guinea pigs injected previously with 30 ml mineral oil. Macrophages were washed with Hanks basic salt solution, the red cells lysed with distilled water, and the cells suspended in RPMI with 10% heat-inactivated guinea pig serum at a concentration of 5 x 10^7 cells/ml. The cells were placed into capillary tubes as previously described (13), and each capillary tube was placed in a 16 mm well of a 24 well tissue culture plate (Linbro, Inc.). One ml of reconstituted MIF-containing or control supernatant was added to each well. All assays were run in triplicate. Migration was measured with a projecting microscope after 24 hours incubation at 37°C in a humidified 5% CO_2 atmosphere. The area of migration was calculated with a planimeter, and the percent migration was calculated by the following formula:

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

RESULTS

Skin tests.

Skin test responses to both PPD and SK were characterized by induration with little erythema. The response was specific against the immunizing antigen with no induration elicited by testing with another antigen (ovalbumin).

The maximal response was noted at 32 hours (Figure 1). Biopsy of the skin test site performed at 30 hours showed mononuclear cell infiltration and moderate edema in the dermis (Figure 2). Skin tests continued to be positive to the same degree up to 6 weeks following immunization; further skin tests were not done after that time.

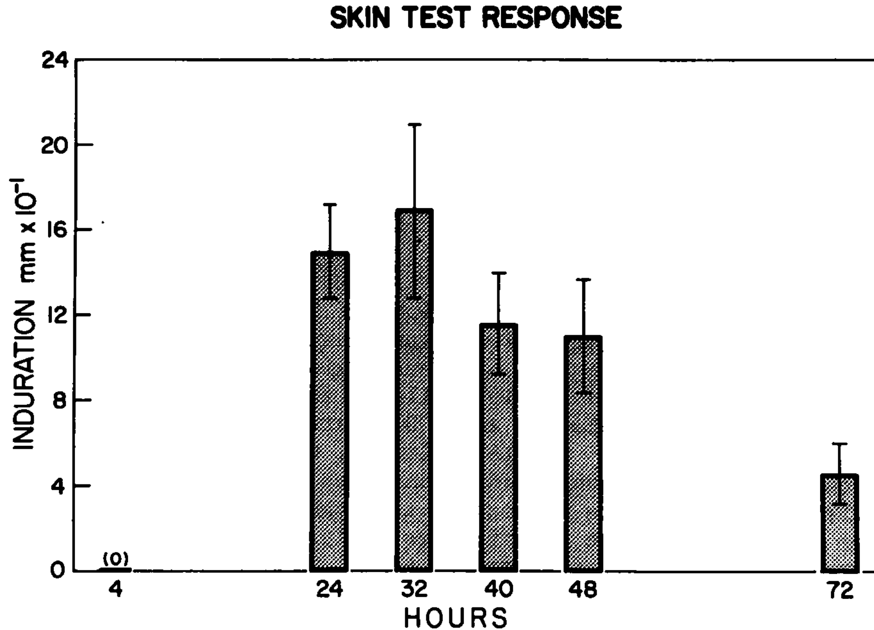

FIG. 1

Skin test response to PPD in 22 ferrets immunized with complete Freund's adjuvant. The top of each bar represents the mean difference in induration between the skin test site and normal skin; the brackets show the standard error of the mean.

Lymphocyte transformation.

The effect of three different sera on mononuclear cell proliferation *in vitro* was tested using peripheral blood lymphocytes from 12 different ferrets (Table 1). Proliferation was supported by human, ferret, and fetal calf serum. After 3 days in culture, trypan blue dye exclusion showed $\geq$ 90% viability with all three sera, but by day 7 over 50% of the cells in cultures with ferret serum were dead. Cultures with either fetal calf serum or human serum showed 15% to 20% dead cells by the end of the 7th day. Because of its commercial availability and the consistent values obtained for non-stimulated cultures using this serum, fetal calf serum was employed in all further studies.

Peripheral blood mononuclear cells from 10 different ferrets were incubated from 2 to 5 days for mitogens and from 4 to 8 days for specific antigens (Figure 3). The dose of mitogen or antigen used was that giving the maximal response (see Figure 4). Mitogenic responses were maximal at

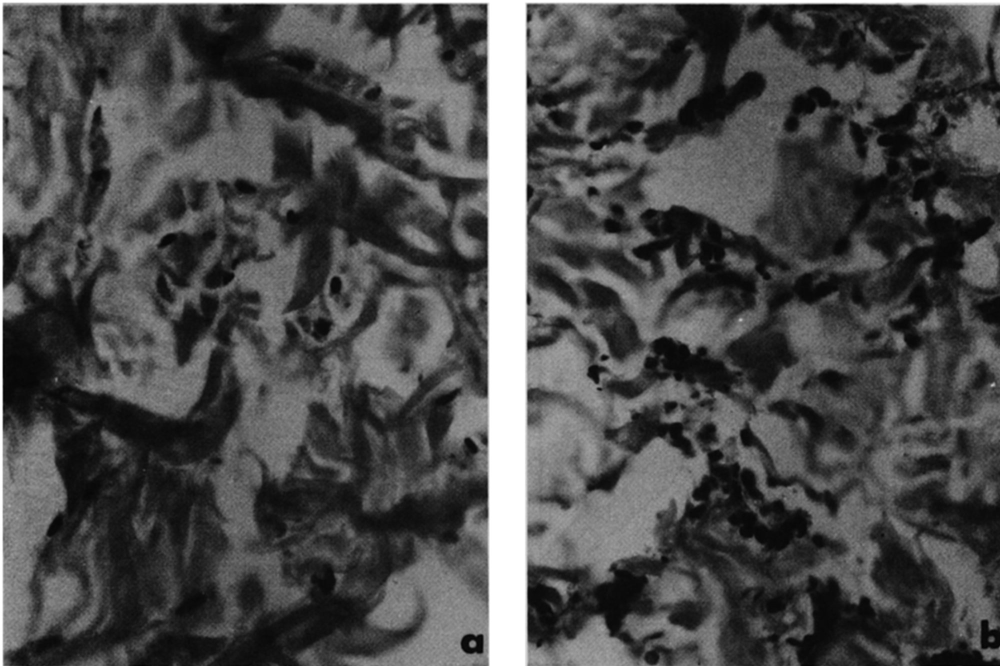

FIG. 2

Biopsy of normal ferret dermis (a) and PPD dermal test site (b) 30 hours after injection of antigen. (x 100).

TABLE 1

Ferret Peripheral Blood Lymphocyte Transformation
Effect of Various Sera on 3-Day Cultures

	Counts per minute x 10^{-3} ($\bar{x}$ ± s.e.m.)		
Mitogen	Ferret	Human	Calf
None	0.2 ± 0.04	0.2 ± 0.05	0.6 ± 0.02
PHA (1:10)	33.3 ± 9	27.4 ± 7	28.2 ± 8
CON A (2.5 µg)	70.7 ± 16	42.9 ± 14	43.1 ± 19

day 2 for CON A, day 3 for PWM and on day 2 or 3, depending on the ferret used, for PHA. Proliferative responses against the antigen SK were maximal at day 5. Background counts varied between 298 and 702 CPM over the 2 to 5 day incubation period for experiments with mitogens and between 226 and 663 CPM over the 4 to 8 day incubation period for experiments with SK. Spleen

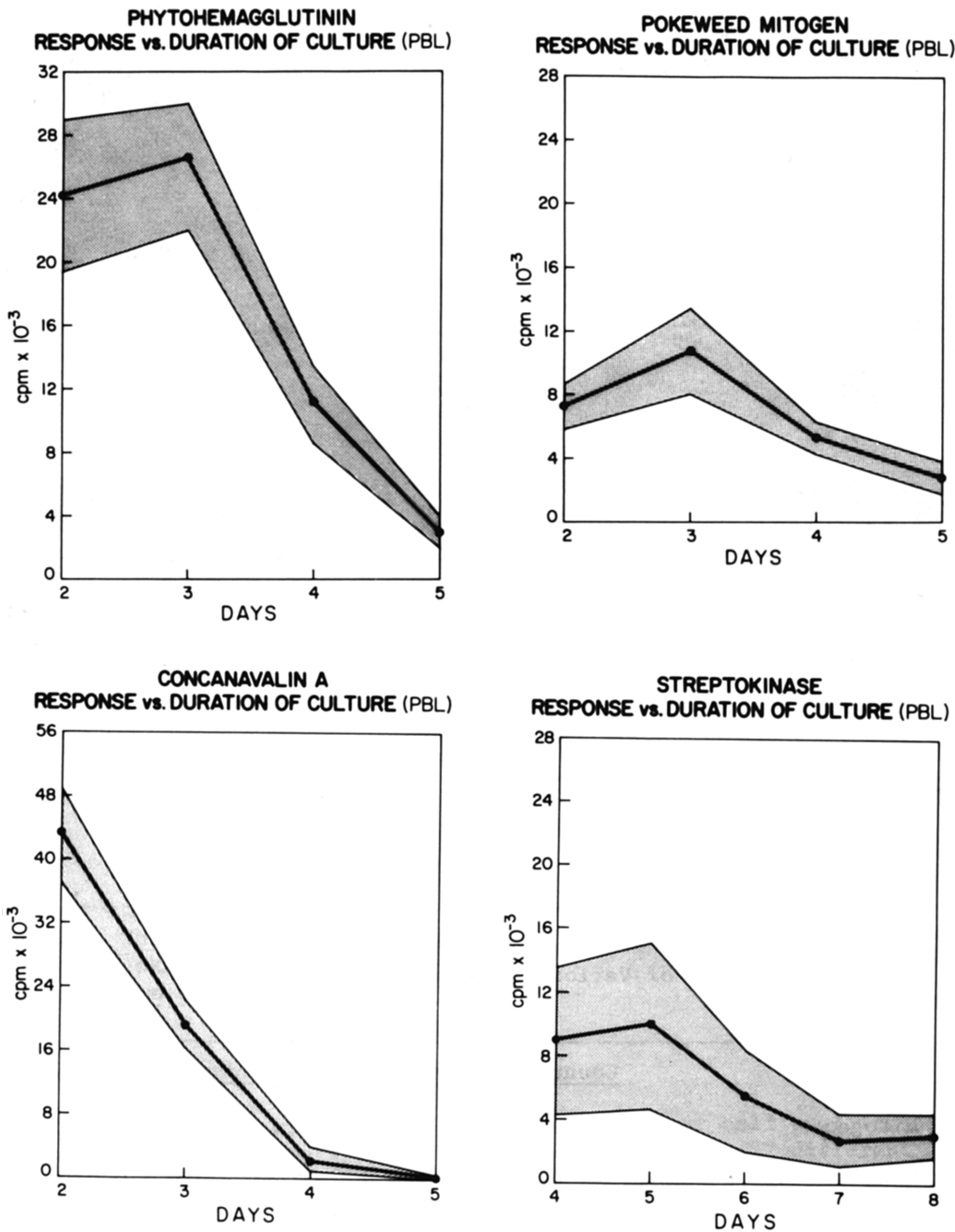

FIG. 3

The effect of incubation time on the proliferative response of peripheral blood lymphocytes to three mitogens, PHA, PWM, and CON A, and the antigen SK. Mean CPM $\pm$ standard error of the mean (shaded area) for 10 ferrets.

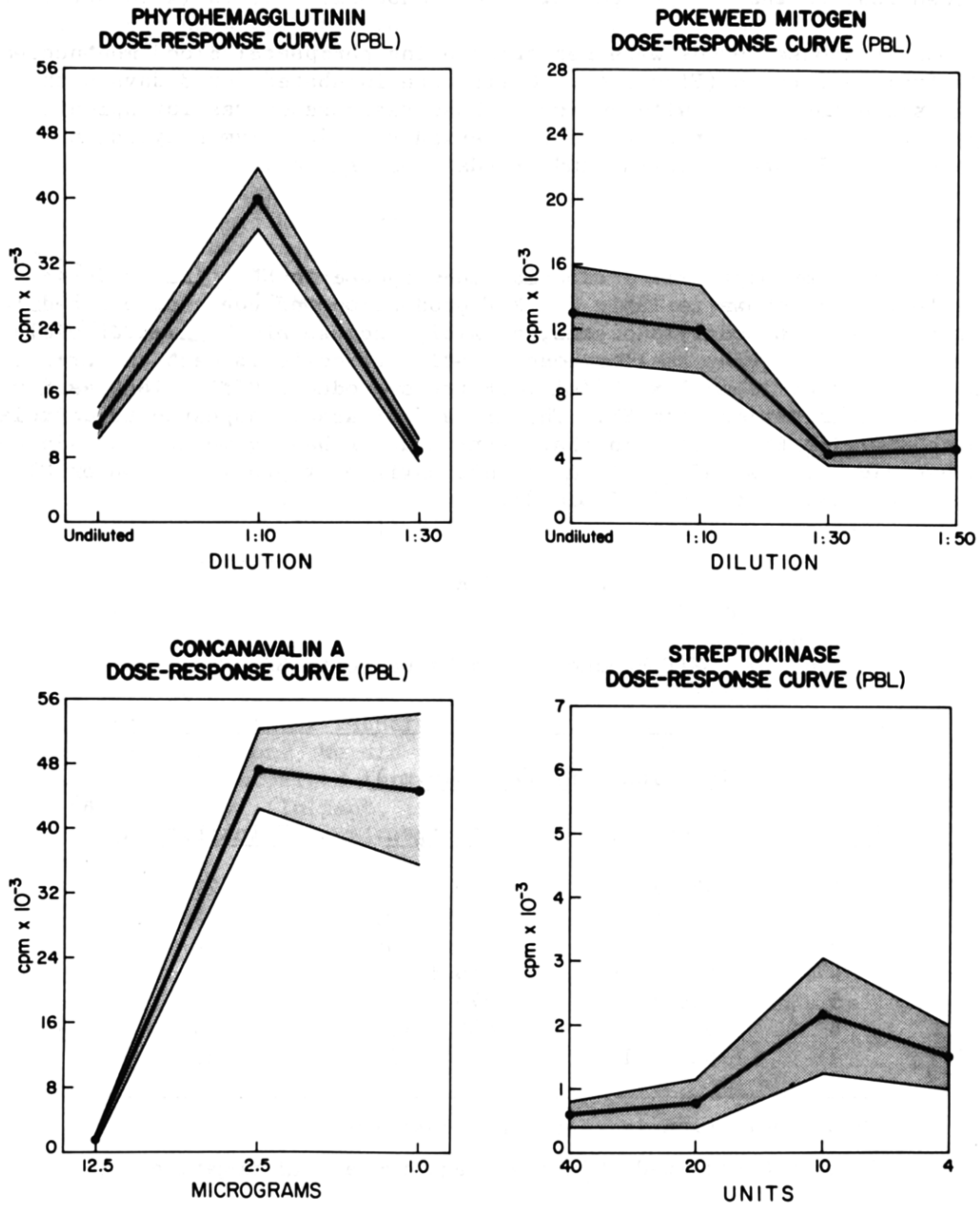

FIG. 4

The effect of concentration on the proliferative response of peripheral blood lymphocytes to three mitogens, PHA, PWM, and CON A, and the antigen, SK. Mean CPM $\pm$ standard error of the mean (shaded area) for 15 ferrets.

cell cultures showed a time-dependent response to mitogens and the antigen, SK, identical to that of peripheral blood mononuclear cells (data not shown).

Dose-response curves were performed using peripheral blood mononuclear cells from 15 ferrets (Figure 4). Cells were incubated for 3 days with mitogens and for 5 days with antigen. Dose-response curves for splenic lymphocytes were similar to those for peripheral blood lymphocytes in response to all three mitogens and SK (data not shown).

MIF assay.

Splenic lymphocyte MIF production in response to SK stimulation in seven ferrets is shown in Table 2. MIF production was not accomplished using peripheral blood lymphocytes, possibly because of the smaller yield of cells and consequent smaller concentration of cells in each culture (5×10^6/ml rather than 1×10^7/ml). Ferrets produced MIF as long as 8 weeks following immunization with SK. The MIF and LT assays appeared to correlate with the *in vivo* DTH assay, in that ferrets never having mounted a skin test response did not respond *in vitro* to that antigen by proliferation or MIF production (e.g. ferret 6 in Table 2).

TABLE 2

MIF Production in Response to SK

	Migration area ($\bar{x} \pm$ s.e.m.)		
Ferret	Control	Antigen	% Inhibition [a]
1	437 ± 17	320 ± 25	27
2	466 ± 116	336 ± 109	28
3	526 ± 100	296 ± 158	44
4	555 ± 39	419 ± 35	25
5	506 ± 60	373 ± 137	26
6 [b]	574 ± 33	489 ± 115	15
7	628 ± 13	472 ± 81	25

[a] >20% inhibition is considered positive

[b] Ferret 6 remained skin test negative and LT negative

DISCUSSION

Classic tests of cell-mediated immunity can be reproduced in ferrets. Previous studies in ferrets have employed one or two tests of cell-mediated immunity but have not dealt with interrelationships between various tests and the reproducibility of each test. Delayed dermal hypersensitivity responses have been difficult to obtain in ferrets, especially when compared with the reactions easily elicited in guinea pigs and humans (8,14); in this regard, ferrets are similar to rats (15). Footpad swelling after injection of antigen, as commonly used in mice as an *in vivo* correlate of

cellular immunity (16), proved to be not reproducible in ferrets. Perhaps this failure can be attributed to the larger amounts of subcutaneous tissue in the footpads of ferrets when compared with the footpads of mice.

Lymphocyte transformation techniques used to study human cells were directly applicable to ferret lymphocyte cultures. Cell death in cultures with fetal calf serum, noted previously with mouse lymphocytes (17), was not a problem using ferret cells. Studies assessing the mitogenic response vs. duration of culture were similar to those found in rabbits and guinea pigs (17). Peak transformation responses were similar to those observed in earlier experiments using unseparated peripheral blood leukocytes from ferrets (9). Splenic lymphocytes responded as well as peripheral blood cells using the same culture conditions, a result at variance with a previous report in which ferret splenic lymphocytes responded poorly to mitogenic stimulation (10). Preliminary evidence using a technique which separates out complement receptor-bearing cells indicates that spleen cells responding to PHA and CON A are non-B cells and may be "T" cells (Kauffman, unpublished data). Using nylon wool separated cell populations, McLaren noted a greater response to PHA in the "T" enriched population (10). Verification of these preliminary results will await more complete identification and separation of ferret lymphocyte subpopulations.

MIF production by ferret spleen cells has been documented once previously (8). Our data agree closely with those of Potter et al. who used an indirect MIF assay similar to ours (8). However, we documented MIF release as late as 8 weeks following immunization, whereas Potter's study showed MIF production to be rather evanescent, with no response shown 3 weeks after immunization. We did attempt a direct MIF assay using peritoneal cells of immunized ferrets; however, this was not as reproducible as the indirect assay. In fact, it was difficult to obtain enough macrophages from the peritoneal cavity of ferrets; the predominant cell type was the neutrophil.

Ferrets do mount cell-mediated immune reactions which are reproducibly measured by *in vivo* and *in vitro* tests. Use of these assays should allow further definition of the role of the cellular immune system in the pathogenesis of certain viral infections for which the ferret is an excellent experimental model.

ACKNOWLEDGEMENTS

The expert technical assistance of Alice Bergman is gratefully acknowledged.

This work was supported by a grant from the Veterans Administration.

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