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In Vitro Production of Feline IgG: Quantification by an Enzyme-Linked Immunosorbent Assay (ELISA)[1] (42718)

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Abstract. We report here on the development of a sensitive and convenient enzyme-linked immunosorbent assay (ELISA) for feline IgG by using commercially available reagents and optimizing their concentrations. The reagents employed include goat anti-cat IgG antibody and alkaline phosphatase-conjugated goat anti-cat IgG antibody. The assay described is sensitive, reproducible, and highly specific for feline IgG. The assay was applied for the measurement of feline IgG synthesized and secreted *in vitro* by peripheral blood mononuclear cells cultured with or without a polyclonal B-cell activator. The amounts of secreted IgG in the supernatants measured by an ELISA correlated well with the numbers of IgG-secreting cells which were induced upon stimulation with pokeweed mitogen and determined by a reverse hemolytic plaque assay.

It has been shown that feline leukemia virus (FeLV), an ocogeneic retrovirus of cats, frequently causes immunosuppression (1, 2). However, the precise mechanism of FeLV-induced immunosuppression has not yet been established. Various immunological responses or immunoparameters have been used in an effort to clarify these mechanisms including analysis of blastogenesis (2), complement-dependent cytotoxic antibody (3), and interferon levels (3, 4). A reverse hemolytic plaque assay (RHPA) has been used as a method to assess polyclonal B-cell activation in a human system (5–7). We previously reported that RHPA is adaptable to a feline system using pokeweed mitogen (PWM) as a polyclonal B-cell activator (8). However, this method is of limited usefulness, because the response of peripheral blood mononuclear cells (PBMC) to form plaque-forming cells (PFC) in response to PWM in the feline system is considerably lower than that observed in the human system. Furthermore, this method is time consuming and it is difficult to measure multiple samples at the same time. Thus, herein we describe a method to quantify the IgG synthesized and secreted by feline PBMC in an *in vitro* culture system by using an enzyme-linked immunosorbent assay (ELISA).

Material and Methods. *Animals.* Pet cats were obtained from the Department of Laboratory Animal Medicine, University of South Florida, Tampa, Florida. The cats were periodically checked for infection by FeLV using an ELISA test kit, Leukassay F (Pitman-Moore, Inc., Washington Crossing, NJ), or by an immunofluorescence test (9). For all studies we used cells from cats which were not viremic and which appeared to be healthy.

Lymphocyte preparations. Heparinized blood was obtained by venipuncture from the jugular vein of healthy cats. The PBMC were separated from fresh blood by Ficoll–Hypaque (Pharmacia Laboratories Piscataway, NJ) density centrifugation. The PBMC were washed three times with Hanks' balanced salt solution and resuspended in complete medium: RPMI 1640 medium supplemented with 15% heat-inactivated fetal bovine serum (FBS), 2 m*M* L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO, Grand Island, NY).

Cell culture. Cat PBMC were adjusted to 1 $\times$ 10^6 cells/ml in complete RPMI medium. The cells were dispensed in 0.5-ml volumes into 12 $\times$ 75-mm plastic tubes (Falcon, Ox-

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nard, CA). The cultures were stimulated with 5 μg/ml or PWM (GIBCO) and were incubated for 7 or 10 days at 37°C in a 10% CO_2 atmosphere.

Reagents for ELISA. The following reagents were used: alkaline phosphatase (ALP)-conjugated or unconjugated affinity purified goat anti-cat IgG antibodies which are H- and L-chain specific (Accurate Chemical and Scientific Corp., Westbury, NY); goat anti-cat IgM antibody (Pel-Freez Biologicals, Rogers, AR); bovine serum albumin (BSA), Cohn fraction V (Calbiochem–Behring Corp., La Jolla, CA); and *p*-nitrophenylphosphate disodium (Sigma, St. Louis, MO) as phosphatase substrate.

The assay buffers were 0.05 *M* phosphate buffer, pH 7.4, containing 0.15 *M* NaCl (PBS) and 0.05% Tween 20 (Sigma) in PBS (Tween/PBS). The coating buffer was 0.05 *M* carbonate–bicarbonate buffer, pH 9.7. The substrate buffer was 10% diethanolamine buffer, pH 9.8.

The standards used were chromatographically purified cat, mouse, goat, bovine, human, sheep, rabbit, chicken IgGs (Cappel Laboratories, Malvern, PA) and dog IgG (Miles Laboratory, Elkhart, IN).

ELISA for feline IgG. Many combinations of reagents and variables of time and concentration were tested to establish a satisfactory procedure. In this report, only the optimized procedure is described. All washes were three times with 0.05% Tween/PBS. All incubations were for 1.5 hr at room temperature unless otherwise mentioned. The wells of 96-well flat bottom microtiter plates (Immulon I; Dynatech Laboratories, Inc., Alexandria, VA) were coated with 75 μl of goat anti-cat IgG antibody (5 μg/ml) in coating buffer and left overnight at 4°C. After washing, 250 μl of 1% BSA/PBS was added to each well and incubated to block the unbound sites. After a second washing, 75 μl of standards or samples was applied to each well in duplicate and incubated for 2 hr. After another washing, 100 μl of 1:5000 ALP-conjugated goat anti-cat IgG antibody was added and incubated at room temperature. The plates were again washed and 100 μl of phosphatase substrate (1 mg/ml) in 10% diethanolamine buffer was added. The optical density (OD) of samples was then measured at 410 nm using an MR 600 microplate reader (Dynatech) every 15 min for 1 hr. The data presented under Results show the mean amounts of IgG secreted by 10^6 cells in the original cultures.

Gel filtration. Normal cat serum (0.5 ml) was applied to a 2.5 × 90-cm column of Sephadex 4B (Pharmacia). The column was equilibrated with 0.02 *M* PBS (pH 7.2) and eluted with the same buffer solution. Gel filtrations were carried out at a flow rate of 10 ml/hr and monitored by measurement of absorbance at 280 nm. Fractions of 5 ml each were collected and assayed for measurement of feline IgG by ELISA. Void volumes were determined by using Blue Dextran 2000 (mol wt 2×10^6). Molecular weight was determined by comparison with proteins of known molecular weight, thyroglobulin (669,000) and aldolase (158,000), as standards (Pharmacia). All procedures were performed at 4°C.

Reverse hemolytic plaque assay (RHPA) for IgG-secreting cells. PFC were enumerated by the modified RHPA using an agarose method as described previously (8). Sheep red blood cells were coupled to purified staphylococcal protein A (Pharmacia) by a chromium chloride technique. The data presented represent means of PFC per 10^6 cells in the original cultures.

Results. *Standardization of ELISA for cat IgG.* To determine the optimal concentrations of reagents, microtiter plates were coated with varying concentrations (0.5 to 10 μg/ml) of goat anti-cat IgG antibodies and varying dilutions (1:500 to 1:20,000) of ALP-conjugated goat anti-cat IgG antibodies were tested. The OD of standard cat IgG samples of known concentration was measured every 15 min for 1 hr. Representative data are shown in Table I. The higher the concentrations of both antibodies used, the more rapid were the reactions observed. We chose 5 μg/ml for goat anti-cat IgG antibody, a 1:5000 dilution for ALP-conjugated goat anti-cat IgG antibody, and 1 hr for reaction time to get the maximum measurable OD. Standard curves which were obtained using the optimal concentrations of antibodies described above, are shown in Fig. 1. The standard curve was almost linear between 0 and 300 ng/ml after 1 hr of reaction time. Conse-

TABLE I. DETERMINATION OF OPTIMAL CONCENTRATIONS OF COATING ANTIBODY AND ALP-CONJUGATED GOAT ANTI-CAT IgG ANTIBODY

Reaction time (min)	Coating antibody (μg/ml)	Dilution of ALP-conjugated goat anti-cat IgG antibody					
		1:2500		1:5000		1:10000	
		IgG 0.5	0.05	0.5	0.05	0.5	0.05 μg/ml
30	10	1.851[a]	0.148	0.888	0.091	0.560	0.068
	5	1.555	0.192	0.773	0.120	0.489	0.085
	1	0.754	0.275	0.421	0.172	0.248	0.103
	0.5	0.321	0.134	0.216	0.099	0.130	0.067
	0	0.022	0.023	0.025	0.033	0.033	0.035
60	10	>2.000	0.308	1.905	0.181	1.201	0.128
	5	>2.000	0.417	1.623	0.223	1.037	0.143
	1	1.490	0.593	0.882	0.337	0.515	0.188
	0.5	0.646	0.277	0.437	0.174	0.244	0.099
	0	0.023	0.026	0.023	0.024	0.023	0.028

[a] Average OD in duplicate.

quently, samples were serially diluted two- or threefold and the IgG concentration was determined in this range.

It is important to include a standard curve for each 96-well plate to avoid the influence of minute variation of each plate due to the high sensitivity. PWM or FBS did not interfere this ELISA system (data not shown).

Specificity of ELISA for cat IgG. First, to test the specificity of this assay, we compared the cat IgG concentrations as measured with concentrations detected with IgGs of various species (Table II). This assay was found to be highly specific for cat IgG, although it did detect dog IgG weakly and human IgG very faintly. Next, we investigated whether the assay detected cat IgM, since ALP-conjugated or -unconjugated goat anti-cat IgG antibodies used in these experiments were represented as H- and L-chain specific. Normal cat serum was gel-filtrated and fractions of 5 ml each were collected. Tenfold serial dilutions of samples were applied to the assay (Fig. 2). A single peak (Fr 77-88; mol wt 143,000) of putative IgG was detected, but no peak appropriate to IgM was seen. We next tested a standard cat IgG preparation by ELISA plates coated with goat anti-cat IgG or IgM antibodies (Table III). As is shown in Table III, we could not detect cat IgG at a concentration less than 1000 μg/ml with the anti-cat IgM coated plates. These two experiments support the conclusion that the assay as carried out here is specific for IgG and does not measure IgM.

Comparison of IgG synthesized and secreted into cell supernatants as measured by ELISA and PFC enumerated by RHPA. Cat

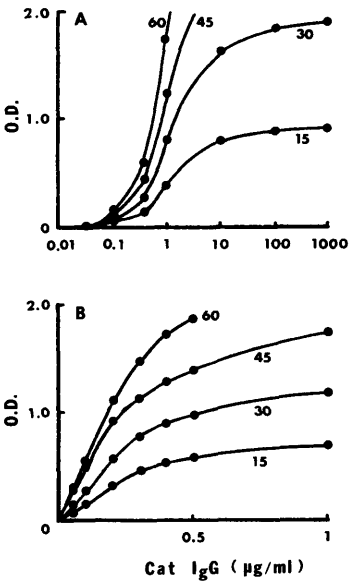

FIG. 1. Standard curves obtained by ELISA using optimally diluted goat anti-cat IgG and ALP-conjugated goat anti-cat IgG antibodies. OD was measured at 15, 30, 45, and 60 min of reaction time as indicated above. Purified cat IgG was applied at the concentration of 0.05–1000 μg/ml (A) and 0.05–1 μg/ml (B).

TABLE II. SPECIES SPECIFICTY OF IgG DETECTED BY ELISA

IgG source	OD at 60 min		
	IgG 0.1	1.0	10.0 μg/ml
Cat	0.570	>2.000	>2.000
Mouse	0.001	0.001	0.006
Bovine	0.001	0.001	0.004
Rabbit	0.001	0.001	0.002
Goat	0.001	0.001	0.001
Dog	0.032	0.147	0.299
Sheep	0.000	0.001	0.001
Human	0.002	0.014	0.053
Chicken	0.001	0.004	0.010

TABLE III. SPECIFIC DETECTION OF CAT IgG BUT NOT IgM

Cat IgG (μg/ml)	Coated with goat anti-cat[a]		
	IgG		IgM
	OD at 30 min	60 min	60 min
1	0.782	1.663	0.003
10	0.964	>2.000	0.004
100	1.367	>2.000	0.005
1000	1.422	>2.000	0.064

[a] Plates were coated with goat anti-cat IgG or IgM antibodies overnight.

PBMC were cultured with or without 5 μg/ml PWM for 7 days. The cultured cells were assayed for IgG-producing cells by RHPA technique and supernatants from the same cells were used to measure the concentration of IgG synthesized and secreted by the cells (Table IV, Expt. 1). The amount of IgG secreted was 610 ng/10^6 cells, whereas the IgG-PFC developed were approximately 200/10^6 cells, after PWM stimulation.

Next, to examine the correlation of PFC and secreted IgG, data from RHPA and ELISA were compared in a kinetic study from Days 3 to 10 using both cells and supernatants from this culture system. The amounts of IgG secreted as measured by the ELISA and the numbers of PFC determined by the RHPA were correlated well from Days 3 to 7 before reaching the peak of PFC response (Fig. 3; correlation coefficient $r = 0.911$, $P < 0.001$, $n = 20$). However, after the peak of PFC response, the numbers of PFC rapidly decreased, whereas the amounts of IgG secreted into the supernatants did not decline on Day 10 (Table IV, Expt. 2).

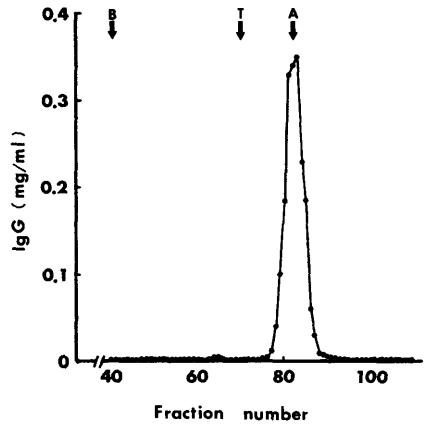

FIG. 2. Gel filtration of normal cat serum on Sephadex 4B column. Standards indicated by arrows are Blue Dextran 2000 (B; mol wt 2×10^6), thyroglobulin (T; mol wt 669,000), and aldolase (A; mol wt 158,000).

Discussion. The double antibody sandwich ELISA described here for cat IgG can be used for the measurement of synthesis and secretion of cat IgG by PBMC *in vitro*. This assay is quick, simple to perform, sensitive, and specific for cat IgG, and it utilizes commercially available reagents. The optimal dose for reagents was found to be 5 μg/ml for goat anti-cat IgG antibody and 1:5000 dilution for ALP-conjugated goat anti-cat IgG antibody. The amount of secreted IgG determined by ELISA correlated closely with data from the RHPA even prior to reaching the peak response in PFC response to a polyclonal activator. Furthermore, IgG in the supernatants was sometimes detected by ELISA and was increased even in the initial few days of culture when only a small number of PFC could be detected (Fig. 3). This finding, suggesting that mature B cells are present in the original PBMC sample and are lost after an initial few days of culture, has been reported by Saxon *et al.* (10). The product of these cells is thus detected in the ELISA but cannot be measured in the RHPA unless kinetic studies are done. Furthermore, after the peak response of PFC is reached, which occurs after ap-

TABLE IV. IgG LEVELS IN SUPERNATANTS AND PFC OF CAT PBMC CULTURED WITH PWM FOR 7 OR 10 DAYS

Expt.	Culture period (days)	PWM	PFC/10^6 cells	IgG (ng)/10^6 cells
1	7	−	33 ± 12[a]	250 ± 42[a]
		+	202 ± 25	610 ± 108
2	7	+	265 ± 36	522 ± 46
	10	+	48 ± 9	552 ± 46

[a] Mean ± SE.

proximately a 7-day incubation, the number of PFC decreases, whereas secreted IgG in the supernatants did not decline (Table IV).

To assess B-cell responses after polyclonal activation, RHPA and ELISA have both been commonly used in the human system (7). Since it has now become extremely important to evaluate B-cell responses and T–B interactions also in cats under many different conditions, establishment of conditions for both of these assays for cats seems highly desirable and useful. In earlier work, Engelman *et al.* (8) showed that RHPA is adaptable to a feline system and the IgG-secreting cells are readily measurable. However, a number of these responding cells in PBMC of cats were found to be very low compared to values obtained for humans. They reported that the number of IgG-secreting cells induced at 7 days of incubation with PWM in the feline system was 235 (range 0–800)/10^6 cells. In a human system, it was reported that IgG-PFC were 4645 (730–11,285)/10^6 cells (6). Unlike in human, PWM could not induce enough polyclonal B-cell activation in cats. The amount of IgG secreted by cat PBMC after 7 days of incubation with PWM was 610 ng/10^6 cells as determined by ELISA (Table IV). This was reported 1641 (672–5702) ng/2 × 10^6 cells using a radioimmunoassay (11) and 975 (390–4020) ng/10^6 cells using an ELISA (12) in the human system. The kinetics of RHPA (6) and ELISA/radioimmunoassay (7) in human system showed a similar pattern until PFC responses reached the maximum. Whereas the number of feline PFC induced by PWM was much lower than that of human, the amount of feline IgG secreted was slightly lower than that of human. The question could be raised whether this discrepancy is attributable to a deficiency of the method used for RHPA in the cats or representative of a difference between the two species. However, the adaptation of the ELISA assay of IgG synthesized and secreted by PBMC in cats has permitted comparative analysis of this function by human and cat PBMC. These studies revealed lower levels of total IgG synthesized and secreted by cat PBMC and support the conclusion permitted by analysis using RHPA that cat PBMC produce less PFC than humans. However, the total IgG produced by the cat PBMC was closer to the values produced by the human cells than seemed to be the case from RHPA. To understand these relationships completely will require extensive kinetic studies using both methods and methods to assess cell viability and durability in the two systems. This issue illustrates clearly the value of having available the new adaptation described in this report. Thus each method may be able to contribute to an understanding of immunocyte functions. This ELISA procedure can be used to determine the amounts of immuno-

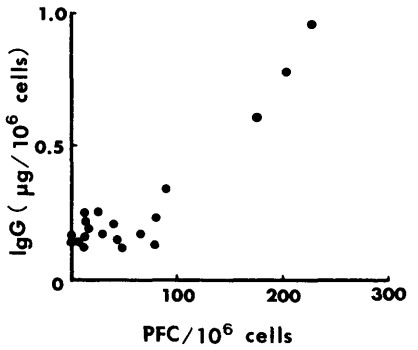

FIG. 3. Correlation between the amounts of IgG secreted into the supernatants measured by the ELISA method and the numbers of PFC determined by the RHPA.

globulin or antibody secreted into the media throughout any time period of the culture, whereas a RHPA can be used to determine immunoglobulin production at a specific time in the culture period.

Using the cat as an animal on which to focus such immunological analysis is particularly attractive because cats are susceptible to influences of oncogenic retroviruses which produce immunodeficiency syndromes and also frequently are associated with evidence of polyclonal B-cell activation and autoimmunity (1). The study of cats and cat lymphoid cells may thus help in unraveling the immunodeficiencies and immunologic perturbation that accompany virus-induced malignancies in general. Furthermore, since a lenti-retrovirus infection recently described in cats (13) is highly T-lymphotropic and morphologically similar to but antigenically distinct from human immunodeficiency virus, study of the immunologic functions in this species under conditions of a variety of naturally occurring and experimentally induced infections requires the availability of incisive methods of immunological analysis in this species.

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