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A Humoral Suppressor of *in Vitro* Lymphocyte Transformation Responses in Cattle with Johne's Disease (38016)

D. H. DAVIES,[1] L. CORBEIL, D. WARD, AND J. R. DUNCAN
(Introduced by J. A. Baker)

Veterinary Virus Institute and Department of Large Animal Medicine Surgery and Obstetrics, New York State Veterinary College, Ithaca, New York 14850

A generalized depression of delayed hypersensitivity responses to various protein antigens and haptens has been demonstrated in human patients with lepromatous leprosy (1). This anergy is associated with the presence of plasma factors which inhibit the blastogenic transformation of lymphocytes by phytohemagglutinin (PHA) and a number of antigens (2, 3). Cutaneous anergy and blastogenic inhibitory factors have also been demonstrated in patients with other chronic disseminated infections, e.g., histoplasmosis (4), secondary syphilis (5), tuberculosis and candidiasis (6), and in patients with nonlymphoid tumors (7).

The purpose of the present study was to investigate the *in vivo* and *in vitro* immunological responsiveness of natural cases of Johne's Disease (JD), a chronic infection of cattle with the intracellular parasite *Mycobacterium johnei.*

Materials and Methods. Animals. The animals studied in this investigation were clinical cases in the large animal hospital of the New York State Veterinary College, Ithaca, NY. Two mature Angus cows, Nos. 7842 and 6896, had clinical histories of progressive emaciation with intermittent diarrhea. Both cows had positive complement fixation titer for JD. Cow No. 6896 had a mild hypoproteinemia (total protein 5.0) while animal 7842 had a mild anemia (PCV 23). This cow was transfused with 4 liters of whole blood two months before this study. No eosinophils were detected in the peripheral blood of cow No. 7842 on repeated hemograms. Other hematological parameters were normal in both cows. Johne's disease was confirmed in both animals either on fecal culture, rectal and/or intestinal biopsy. Control animals used in this study were normal Holstein cows which were part of the University herd.

Immunization, Skin Testing and Serological Procedures. Cow 7842 was immunized by the administration of a total of 4 ml of killed *Brucella abortus,* strain 19 and *Mycobacterium tuberculosum* incorporated into incomplete Freund's adjuvant. Cow 6896 was immunized with 4 ml of *Vibrio fetus* and *Mycobacterium butyricum* in incomplete Freund's adjuvant. Serum samples were taken prior to immunization and at weekly intervals. Skin testing for a cell mediated response was conducted by inoculating .1 ml of tuberculin and brucellergin (7892) or *Vibrio fetus* antigen (6896) intradermally. Double skin thickness was measured prior to inoculation and at 48 hr. The serology for *Vibrio fetus* (8) and *Brucella abortus* (9) was conducted according to established procedures.

Lymphocyte Transformation. Phytohemagglutinin-induced lymphocyte transformation (LT) responses were measured using modifications of the method described previously (10). Plasma from animals with JD was assayed for inhibitors of LT using whole peripheral blood from normal cattle. The responsiveness of lymphocytes from a case of JD was measured in autologous plasma and in normal bovine plasma. Ali-

[1] Present address: Wallaceville Animal Research Centre, Private Bag, Upper Hutt, New Zealand.

quots of blood from a normal cow (No. 7) and one with JD (No. 7842) were incubated in their own plasma or plasma from the other animal of the pair. When heterologous plasma was used the cells of 3 ml of blood were sedimented (400*g*, 20 min) and the plasma fraction was removed and discarded. The cells then were washed once in heterologous plasma and finally made up to the original volume in the same heterologous plasma. The plasma used for the washing and final dilution of the cells was prepared on the day of the test. Duplicate 0.5 ml aliquots of blood in autologous or heterologous plasma were incubated with equal volumes of PHA solution (PHA-M, Difco, lot No. 573257) as described previously (10). Cultures were labelled with tritiated thymidine (^{3}H Tdr) and processed for liquid scintillation counting as described (10). When the inhibitory activity of serum or serum fractions was measured, the serum or fraction was mixed with an equal volume of McCoy's 5A medium (Microbiological Associates) plus antibiotics. Aliquots of 0.5 ml of heparinized blood from normal cattle were mixed with an equal volume of the McCoy's-serum mixture. Then 0.05 ml of a solution of PHA (10 mg/ml) in PBS (phosphate buffered saline, 0.15 *M*, pH 7.2) or 0.05 ml of PBS was added to the stimulated and control cultures, respectively. The cultures were incubated, labelled and processed for liquid scintillation counting as described before. Sera used in this part of the investigation were collected from the 2 animals with JD (Nos. 7842 and 6896). The inhibitory activity of these sera was assayed in duplicate cultures on 5 occasions using blood from 4 different normal cows. The uptake of ^{3}H Tdr by lymphocytes from the normal cattle incubated in normal bovine serum was used for the calculation of inhibitory activity. Inhibitory activity was expressed as % inhibition where

$$\% \text{ inhibition} = \frac{\text{net cpm in normal serum} - \text{net cpm in test serum}}{\text{net cpm in normal serum}} \times 100$$

and Net cpm = cpm PHA stimulated cultures − cpm unstimulated cultures.

Ion Exchange Chromatography. Serum from cow No. 7842 and a control cow No. 7 were fractionated on DEAE Sephadex A-50 using a linear gradient from

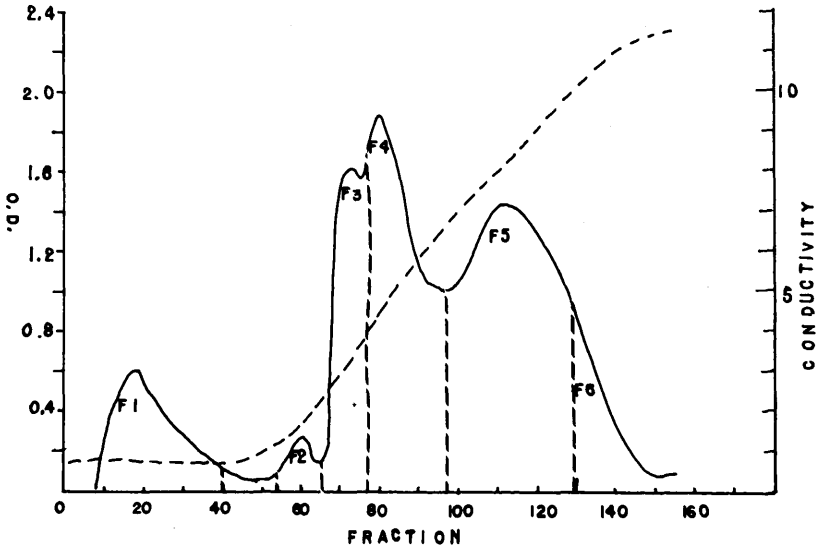

FIG. 1. Ion exchange chromatogram of bovine serum from JD cow No. 7842. F 1 to 6 represent pools of serum fractions—O.D. read at 280 and conductivity in millimhos.

0.01 *M* Tris HCl + 1 m*M* EDTA pH 8.6 to 0.7 *M* Tris HCl + 1 m*M* EDTA pH 8.6 as previously published (11).

Matched fractions from each chromatogram were pooled, (Fig. 1) dialyzed against distilled water, lyophylized and with the exception of fraction 2, reconstituted to approximately 20 mg of dried protein per ml of PBS pH 7.2. These 6 fractions were dialyzed for 48 hr against PBS, sterilized by filtration and used in the lymphocyte culture medium as described above.

The immunoglobulin distribution of each fraction was determined by micro gel diffusion using monospecific antisera against bovine immunoglobulins G_1, G_2, A and M and α_2 macroglobulin.

The inhibitory activity of these fractions was assayed in duplicate cultures of blood from 2 normal cows. The percent inhibition of each fraction was calculated by comparing the net uptake of ^{3}H Tdr in fractions of JD serum with the net uptake in the equivalent fractions of normal serum.

Histology. Lymph nodes, thymus, distal ileum and other major organs were placed in 10% formalin, processed by standard techniques, sectioned at 6 μm and stained with H & E.

Results. When peripheral blood cultures from a cow with JD were incubated in autologous plasma, the PHA-induced ^{3}H Tdr uptake was poor in comparison with the response of cultures from a normal cow at the same stage of gestation. However, when the cells from either of these animals were washed and incubated in plasma from the opposing animal of the pair the situation was reversed. The cells from the JD-affected animal now responded normally whereas the uptake of ^{3}H Tdr by normal cells in JD plasma was decreased by 99% (Table I). Sera from this animal and another animal with JD also caused significant suppression of the PHA-induced blastogenic response when they were incorporated in the culture medium (Table II). The presence of the serum immunosuppressive factor correlated with the inability of cow No. 7842 to give a delayed hypersensitivity skin reaction to tuberculin after the inoculation of *Mycobacterium tuberculosum* in incomplete Freund's adjuvant (Table III). Both animals had normal humoral immune

TABLE I. PHA-Induced Tritriated Thymidine Uptake by Peripheral Blood Leucocytes of a Normal Cow and a Cow with Johne's Disease in the Presence of Normal or Johne's Disease Plasma.

	Johne's disease cells #7842	Normal cells #7
Autologous plasma[a]	3116[c]	10033
Heterologous plasma[b]	10241	124
% Change	70%	99%

[a] Blood incubated in animal's own plasma.

[b] Blood washed and incubated in opposing animal's plasma.

[c] Mean counts per minute, 4 replicate cultures.

TABLE II. Effect of Serum from Cattle with Johne's Disease on PHA-Induced Tritiated Thymidine Uptake by Normal Peripheral Blood Lymphocytes.

Source of test serum[a]			
Animal No.	Condition	CPM ± SE[b]	% Inhibition[c]
7	normal	17024 ± 3145	—
6896	Johne's disease	8845 ± 1630[d]	48
7842	Johne's disease	3253 ± 1466[d]	80

[a] Serum used in blood culture medium with cells from 4 different normal cattle.

[b] Uptake of tritiated thymidine, net counts per minute, mean of 10 replicates ± 1 standard error.

[c] $\frac{\text{net cpm in normal serum (No. 7)} - \text{net cpm in disease serum}}{\text{net cpm in normal serum}} \times 100.$

[d] Significant suppression of ^{3}H Tdr uptake in comparison with cultures incubated in normal serum ($P < 0.005$). Student's t test.

TABLE III. Response of Normal and Diseased Cattle to Skin-Testing with Tuberculin Three Weeks after Sensitization with *Mycobacterium tuberculosum*.[a]

Animal No.	Condition	Skin thickness 0 hr	Skin thickness 48 hr
7842	Johne's disease	0.79	0.81
66	Normal	0.69	1.43

[a] Double skin thickness in centimeters.

responses to *Vibrio fetus* or *Brucella abortus* antigens.

The serum proteins from one of the JD cows (No. 7842) were separated by ion exchange chromatography and divided into fractions which were included in the lymphocyte culture medium. The major constituents of these fractions are shown in Table IV. When the uptake of ^3H Tdr by lymphocytes incubated in these fractions was compared with the uptake in the equivalent fraction of normal bovine serum, the inhibitory activity of JD serum was found in fractions 1, 3 and 4 (Table V). Fraction 1 contained only IgG_2 while F 3 and F 4 contained IgG_1 and IgG_2. Inhibitory activity was found in 1 fraction which contained α_2 macroglobulins (fraction 4) but no inhibiting activity was found in F 5 which contained the bulk of α_2 macroglobulin or in fraction 2 which contained low levels of IgG_2.

Gross lesions consistent with Johne's Disease (12) were found in both animals. These lesions were characterized by a thickened, corrugated small intestinal mucosa

TABLE IV. Protein Components of Chromatographic Fractions.

Fraction	IgG_2	IgG_1	IgM	IgA	α_2M[b]
F 1	+	–	–	–	–
F 2	+	–	–	–	–
F 3	+	+	–	–	–
F 4	+	+	–	–	+
F 5	+	+	+	+	+
F 6	+	+	–	–	+

[a] Proteins detected by microgel diffusion on ion-exchange fractions of serum 7842.

[b] α_2 Macroglobulin.

TABLE V. Effect of DEAE-Sephadex Fractions of Normal and Johne's Disease Serum on PHA-Induced Transformation of Normal Peripheral Blood Leucocytes.

Fraction	Counts per minute[a] Normal serum	Counts per minute[a] Johne's disease serum	% Inhibition[b]
1	7703	3011	61
2	6608	6642	0
3	6336	2957	53
4	7715	3529	54
5	8090	9866	0
6	10612	10765	0

[a] Uptake of tritiated thymidine as counts per minute per 0.1 ml blood mean of duplicate cultures from 2 different animals.

[b] % Inhibition =

$$\frac{\text{cpm in normal serum} - \text{cpm Johne's Disease serum}}{\text{cpm normal serum}} \times 100.$$

(ileum) and dilated intestinal lymphatics. No thymus could be found in cow No. 6896 although this could have been overlooked.

Histological examination of the ileum revealed a mild to marked infiltration of the lamina propria and occasionally the submucosa of the small intestine with epithelioid type macrophages and giant cells in which acid-fast bacilli could be demonstrated. Similar granulomatous lesions were present in the intestinal lymphatics and extending to the mesenteric lymph nodes but paracortical areas contained few small lymphocytes with numerous epithelioid type macrophages and the occasional Langhan's type giant cell infiltrating this region (Fig. 2). The thymus of cow No. 7842 had a well defined cortex but the medulla appeared to be deficient in epithelial reticular cells and definable Hassel's corpuscles.

Discussion. An inhibitor of PHA-induced lymphocyte transformation has been demonstrated in the plasma and serum of cattle with JD, a chronic mycobacterial infection. This humoral immunosuppressive factor was associated with the inability of 7842 to mount an effective cell-mediated immune response as measured by delayed hypersensitivity reactions to tuberculin. A further indication of impaired cell-mediated

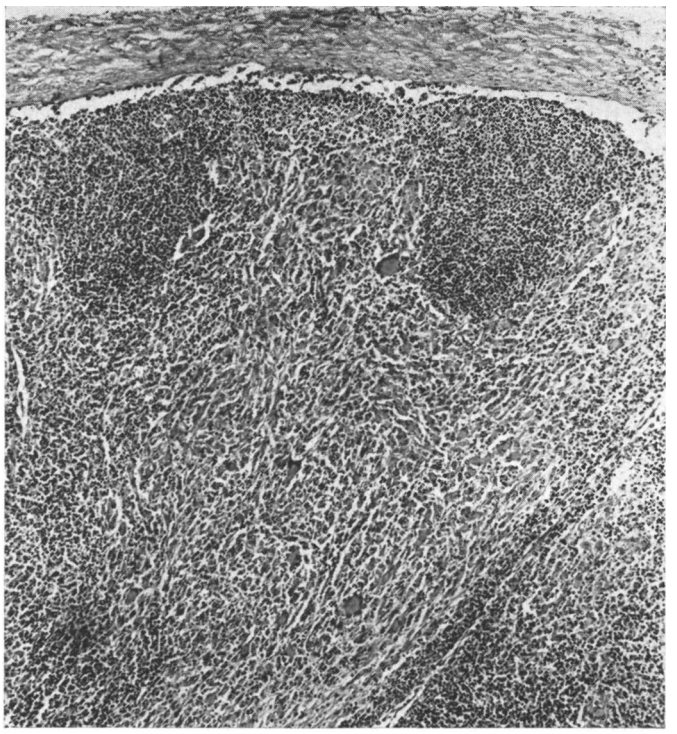

FIG. 2. Mesenteric lymph node from JD cow No. 7847. Infiltration of paracortical area with epithelioid macrophages and Langhans giant cells with loss of paracortical small lymphocytes. H & E × 80.

immunity was the inability of these animals to overcome the chronic intracellular infection.

Inhibitors of *in vitro* lymphocyte transformation have been demonstrated in plasma or serum from human patients with chronic intracellular infections (6) and nonlymphoid tumors (7) but few of these factors have been characterized. The inhibitory factor in serum from cases of lepromatous leprosy was shown to be heat stable and nondialyzable but was not characterized further (2). In patients with primary intracranial tumors the immunosuppressive activity was found in the IgG fractions of the serum (7) however, in colonic cancer patients, plasma inhibitory effects were correlated with the levels of serum carcinoembryonic antigen and α globulin (13). Human α-fetoprotein from cord plasma (14), α-fetoprotein from rats with hepatomas (15) and α-globulin fractions of normal human plasma (16) have also been shown to have *in vitro* immunosuppressive properties. In this investigation the inhibitory factor in JD serum was found in the IgG rich fractions one of which contained only IgG_2. Inhibition was not demonstrated in fractions containing the bulk of α_2 macroglobulin and in fact cultures incubated in these fractions of JD serum had increased uptake of ^3H Tdr in comparison with control cultures. However, inhibitory activity in serum from cattle with tuberculosis was considered to reside in the α_2-globulin fraction (17).

The recovery of the suppressor activity in the IgG containing fractions suggests that the suppressor may be an antibody but its specificity or mode of action was not determined. However, in autologous systems at least, the antibody must be loosely bound to the lymphocyte and not lymphocytoxic since the cells of No. 7842 responded normally after a single washing in normal plasma. In other species there is evidence

that these inhibitory factors are auto-antibodies which bind loosely to the potentially reactive lymphocytes preventing antigen recognition or binding of phytomitogens and thus function as a feed back control of cell-mediated immunity to prevent excessive tissue destruction (6). This may be their role in chronic intracellular infections such as JD.

In humans with lepromatous leprosy, the immunodeficient form of the disease, the paracortical areas of the draining lymph nodes are infiltrated with undifferentiated cells of the histiocyte–macrophage series (18). Whereas, in tuberculoid leprosy where cell-mediated immune responses are often normal, the paracortical areas of the lymph nodes are well developed and populated with lymphocytes. Lymph node changes similar to those reported in lepromatous leprosy were seen in the cattle with JD in this study and, as in leprosy, these lesions were associated with cutaneous anergy and the presence of humoral immunosuppressive factors (1, 2, 18).

Thus it appears that the destruction of the paracortical areas of lymph nodes, the inability to mount cell-mediated immune responses with the concomitant elimination of the infection and the presence of humoral immunosuppressive factors may be a general feature of chronic intracellular infections in animals as well as man. If this holds true, Johne's disease of cattle may provide a useful model to investigate the role of cell-mediated immunity and immunosuppressive serum factors in the pathogenesis of chronic intracellular infections.

Summary. Cattle with Johne's Disease, a chronic mycobacterial infection, often have a cutaneous anergy. The lymphocytes from a Johne's Disease affected animal responded poorly to phytohemagglutinin (PHA) when incubated in autologous plasma. When the cells were washed and incubated in normal plasma, the PHA responses returned to normal values. Sera from these cattle also were found to inhibit PHA-induced transformation of normal peripheral blood lymphocytes. When the immunosuppressive serum from one of these animals was fractionated by ion-exchange chromatography the suppressor of lymphocyte transformation was recovered in the fractions containing immunoglobulins G_1 and G_2.

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