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Immunological Studies in Cattle Exposed to Polybrominated Biphenyls

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The intactness of the immune system in cattle exposed to polybrominated biphenyls (PBBs) has been investigated by using several immunoassays. Eighty-seven animals have been studied, 35 control animals (not exposed to PBBs) and 52 animals exposed to PBBs (0.02–30 ppm/g fat equivalent). The immunoassays included a complete blood count, identification of peripheral blood T and B lymphocyte subpopulations, serum immunoglobulin levels (IgG, IgM, and IgA), the *in vitro* response to lymphocytes to phytolectins (PHA, Con A, PWM), the antibody response to Keyhole limpet hemocyanin (KLH), the cell-mediated response to PPD, and determination of autoantibodies and/or immunosuppressive serum factors.

For control and PBB-exposed cattle, there was no statistical difference between the number of circulating erythrocytes or leukocytes, the hematocrit, or hemoglobin content; the percentage or number of T and B lymphocytes; the isotope incorporation index (DNA synthesis) of lymphocytes in response to mitogens; the concentrations of serum immunoglobulins IgG, IgM, or IgA; the mean peak titer to KLH; or *in vivo* or *in vitro* immune response to PPD.

Additional evaluation of cattle with tissue levels of PBB greater than 3 ppm/g tissue for hematological and immunological parameters revealed no statistical difference from control animals.

Other experiments were performed to evaluate serum from cattle exposed to PBBs for autoantibodies to smooth muscle, mitochondrial or nuclear antigens. No evidence for autoantibodies was observed.

Further studies were done to examine the cytotoxic and/or immunosuppressive activity of sera from PBB-exposed animals. In these studies, the blastogenic response of lymphocytes from control cattle and humans were evaluated in the presence and absence of serum from animals exposed to PBBs (> 3 ppm/g tissue). No evidence for either a cytotoxic or an immunosuppressive influence of such sera was demonstrable.

Our studies indicate that PBB, at the levels studied, does not alter or interfere with lymphocyte surface antigens, the complex nuclear and cytoplasmic events required for mitosis and cell division, or the biological events required for antibody formation and cell-mediated immune reactions. Further, PBB exposure at the levels studied does not predispose cattle to autoantibody production or leucotoxic serum factors.

Introduction

In 1973, polybrominated biphenyls (PBBs) accidentally entered the food chain in Michigan when FireMaster, a commercial flame-retardant chemical, was substituted for NeutriMaster, a magnesium oxide used as a supplement in livestock feed (*1*). Approximately 500 to 1000 pounds of PBBs were mixed into feeds that were widely sold and distributed to Michigan farmers. During the months following the accident, some contaminated animals exhibited unusual clinical and pathological signs. In an attempt to limit human consumption of meat and dairy products from these animals, 30,000 cattle, 1.5 million chickens, and 6000 swine with PBB levels above the Food and Drug Administration's guidelines were destroyed (*2*). Several studies have recently been undertaken to advance our understanding of the consequences of PBB contamination.

Jackson and Halbert (*3*) initially described a toxic syndrome occurring in a dairy herd that received high concentrations of PBBs (up to 3000 ppm per g fat). Toxicity studies have now been reported for several species (*4, 5*). Additional studies have investigated the chemical composition of FireMaster (*6*), the half-life of some of its constituents (*7*), and the solubility of the PBBs (*7*). These studies have established that PBBs are fat-soluble, stable substances which are biomagnified within some tissues, especially adipose tissues. Biochemical studies re-

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veal PBB contamination can effect storage in body lipids (*8*), enzyme systems (*9*), and other cellular metabolic events (*10*). No clear evidence has been reported indicating either a carcinogenic or mutagenic property of PBB (*2*). Additional studies have documented a teratogenetic property of PBB (*2*); however, this finding has not been confirmed. Selikoff and co-workers (*11*) have purported human health effects in farm family members and general Michigan consumers; however, these studies have not been confirmed.

In addition to the toxicosis reported by Jackson and Halbert (*3*), a variety of nonspecific clinical signs have been reported in dairy cattle and other animals with nontoxic levels of PBBs. Infections have been a frequent observation. Frequent and/or prolonged infections often indicate a compromised immune system. Further, necropsy findings in animals receiving toxic levels of PBBs exhibited an atrophic thymus and abnormal lymph node findings (*3*). Moorhead et al. (*12*), in a controlled study, confirmed many of the clinical and histological observations in cattle receiving 820 to 1500 g PBB; however, at lower concentrations (less than 15 g), clinical and histopathological findings were unremarkable.

No studies have yet been forthcoming to evaluate the immune system of cattle exposed to PBBs at any level. The present study was undertaken to investigate the immune competence of cattle exposed to nontoxic concentrations, trace amounts to 30 ppm/g fat. The results indicate that PBB at these concentrations does not alter the immune capacity of the adult bovine.

Materials and Methods

Animals

Eighty-seven cattle, 52 of which were exposed to PBBs have been studied. Of these, 35 animals were obtained from the research farm operated by Farm Bureau Services, Inc., and 15 animals were obtained from the experimental herd at the Ohio Agricultural Research and Development Center. The remaining animals were provided from working herds in Michigan.

PBB Determinations

PBB levels were determined for every animal in this study. Fat biopsies were obtained from all animals in the research herds while fat and/or milk specimens were evaluated for animals obtained from the working herds. PBB determinations were performed by the Michigan Department of Agriculture, Lansing, Michigan, the Wisconsin Alumni Research Foundation, Madison, Wisconsin, or Anatec, Ann Arbor, Michigan. The PBB values are reported as parts per million (ppm) per gram fat.

Blood Specimens

Peripheral blood was obtained by venipuncture. The blood was collected in EDTA or heparin or allowed to clot in glass tubes. Each blood specimen received a coded number and was delivered within 16 hr to the Immunology Laboratory at E. W. Sparrow Hospital. All tests were performed without knowledge of the origin of the specimen, i.e., control or PBB-exposed.

Hematology

Partial blood counts (PBC) which included white blood cell and red blood cell counts, the hemoglobin content, and hematocrit were performed on every animal. Complete blood counts (CBC) including the differential were performed on 35 animals, by using peripheral blood smears stained with Wright's stain.

Lymphocyte Preparations

Lymphocyte preparations for *in vitro* immunological studies were obtained by using Ficoll-Hypaque (F/H) gradient centrifugation. Heparinized blood (10 ml) was mixed with an equal volume RPMI 1640 medium and layered onto a mixture of F/H (sd 1.077) in a 1:1 ratio. Following centrifugation at 400 × g for 40 minutes, leukocytes (greater than 90% lymphocytes) were recovered and washed three times by centrifugation with RPMI 1640 medium. Following the final centrifugation, cell suspensions were adjusted to 2×10^6 nucleated cells/ml.

Lymphocyte Surface Receptors

Lymphocytes recovered from F/H gradients were evaluated for immunoglobulin (sIg), complement (C), or sheep erythrocytes (E) receptors. Surface immunoglobulin (sIg)-bearing cells were enumerated with an FITC-labeled rabbit antibovine Ig reagent. Complement (C)-bearing cells were detected by rosetting procedures using antigen-antibody complement complexes (*13*). Erythrocyte (E) binding cells were also determined using a rosette assay utilizing neuraminidase-treated sheep red blood cells (SRBC). For the rosette assays, 2×10^5 lymphocytes were mixed with 10^7 E or EAC cells, centrifuged at 600 rpm for 6 min, and incubated overnight at 4°C. The cell pellets were gently resus-

pended on the following day and the rosette forming cells (RFC) were determined by counting 200–300 cells.

Quantitative Immunoglobulin Analysis

Immunoglobulin concentrations, IgG, IgM, and IgA, were determined by radioimmunodiffusion procedures. Immunodiffusion kits were purchased from Miles Laboratories, Elkhart, Ind. For IgM and IgA determinations, 10 μl portions of serum were used; to determine the IgG concentrations (mg/dl), 2.5 μl portions of serum were used.

Mitogen-Induced DNA Synthesis

The functional status of bovine lymphoid cells was assessed by blastogenesis procedures (*14*). Leukoagglutinin (LA), Pharmacia, Piscataway, NJ; Concanavalin A (Con A), Calbiochem, San Diego, CA; and Pokeweed Mitogen (PWM), Gibco, Grand Island, NY; were used at varying concentrations to stimulate DNA synthesis in lymphocyte cultures. Leukoagglutinin and Con A were studied over a 20-fold range (0.1 to 2.0 μg/culture) while PWM was studied over a 100-fold range (0.01 to 1.0 μg/culture). Leukocyte cultures (2×10^5) were supplemented with 2.5% heat-inactivated fetal calf serum (Reheis, Chicago, Ill.) and incubated at 37°C for 3 days. Tritiated thymidine (^{3}H-Tdr) was added to each culture at approximately 20 hours prior to culture harvest, i.e., 52 hr following culture initiation. The isotope incorporation index (III) was determined from the ratio of radioactivity in cultures stimulated with mitogen to those cultures without mitogen.

Humoral Immunity

Seven control and seven PBB-exposed cattle (0.04 to 1.8 ppm/g fat) were inoculated IM with 5 mg Keyhole limpet hemocyanin (KLH) emulsified in complete Freunds adjuvant (Gibco). Following immunization, blood was collected at weekly intervals for 5 weeks. Serum from each animal was tested for antibody to KLH by use of tannic acid-treated sheep erythrocytes coated with this protein (*15*). Antibody titers were recorded as the highest serum dilution with positive macroscopic agglutination.

Cell-Mediated Immunity

Five control and five PBB-exposed cattle (0.03 to 0.54 ppm/g fat) were inoculated subcutaneously with $1–5 \times 10^8$ BCG organisms (Research Foundation, Chicago, Ill.) emulsified in incomplete Freunds adjuvant. On the 35th day following sensitization, 0.2 mg purified protein derivative (PPD) Connaught, Laboratories, Toronto, were injected intermally in the neck. After 24 hr, the delayed hypersensitivity skin reaction was measured. The *in vitro* response to bovine lymphocytes to PPD was also measured. Then 2×10^5 lymphocytes were stimulated *in vitro* for 3–7 days with varying doses of PPD. Tritiated thymidine was added approximately 20 hr before the cultures were harvested and the III determined as described above. Similar experiments were conducted on these same animals prior to BCG stimulation.

Serum Studies

Serum from control and PBB-exposed cattle were tested for leukocytotoxic factors by incubating 10^6 bovine or human leukocytes in 1 ml of fresh autologous or allogeneic bovine serum for 2 hr at 37°C. In some experiments, fresh rabbit complement, used in lymphocytotoxicity testing for HLA antigens, was added to leukocyte suspensions in bovine serum. The cytotoxicity index was determined by counting 200 cells following trypan blue staining. Evaluation of serum for antinuclear, mitochondrial, and smooth muscle autoantibodies was also included in these studies. Cryostat-cut sections from rat liver, kidney, and stomach were incubated with serum (1/5 dilution) from control or PBB-exposed animals for 30 min at room temperature. The sections were washed in phosphate-buffered saline (PBS), pH 7.4, and stained with fluoroscein-labeled antibovine immunoglobulin serum. The staining reaction continued for 30 min. The sections were rinsed in PBS and examined for fluorescence by using a Zeiss fluorescent microscope.

Statistical Analysis

The data obtained from control and PBB-exposed animals were evaluated for statistical significance by using the Student *t* test. The data obtained from control animals ($N = 35$) were compared to those obtained from all PBB-exposed animals ($N = 52$) and to those obtained from animals with PBB levels exceeding 3 ppm/g fat ($N = 13$). The mean and the standard error are reported on all data collected. A p value less than 0.05 was considered significant.

Results

Hematology

Results from hematological studies are presented in Figure 1. No statistical differences were observed

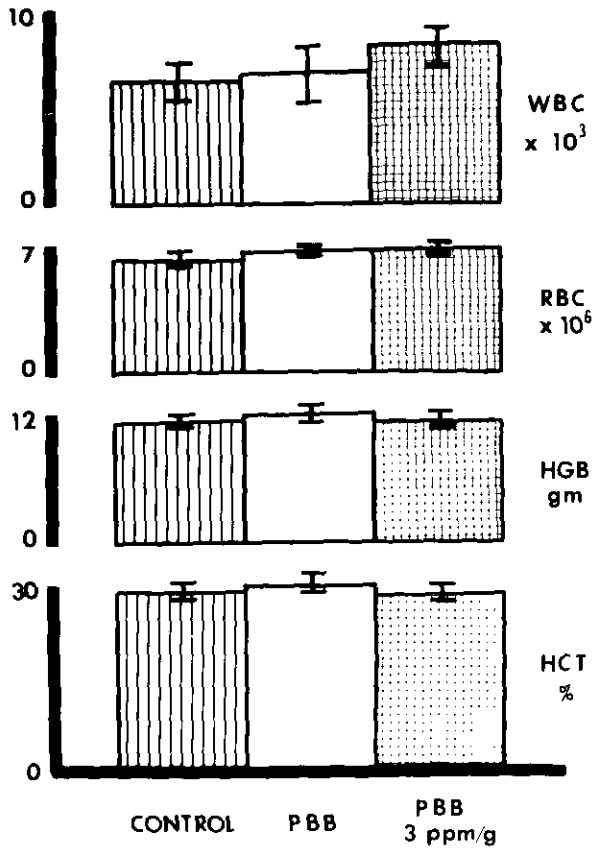

FIGURE 1. Hematological profiles in control and PBB-exposed cattle.

in the WBC or RBC counts, the hematocrit or hemoglobin content between control and PBB-exposed animals. Similarly, animals with body burdens greater than 3 ppm/g fat showed no statistical differences from control values. Differential cell counts to determine the lymphocyte content of peripheral blood also revealed similar percentages in control and PBB-exposed animals (Table 1).

Table 1. Peripheral blood differential cell analysis.

Experimental group	Segmentals, %[a]	Lymphocytes, %[a]
Control (15)[b]	36.8 ± 2.9**	49.1 ± 2.0
PBB (20)[b]	40.1 ± 3.0	47.7 ± 3.5

[a] Mean ± standard error.
[b] Number in parenthesis indicates number of cattle studied.

Additional studies were performed to further characterize bovine lymphocyte populations based on surface membrane characteristics. Lymphocytes spontaneously binding sheep erythrocytes (E), antigen-antibody complement complexes (EAC), or those containing surface immunoglobulin (sIg) receptors were enumerated. The results of these studies are shown in Figure 2. The percentage E lymphocytes in control cattle varied between 7.5 and 29% with a mean of 16.7 ± 1.0%. This lymphocyte population in PBB-exposed animals varied between 7 and 33% with a mean of 15.9 ± 0.6%. A similar range was observed in animals with PBB levels greater than 3 ppm/g fat (mean 15.8 ± 1.37%). The percentage of EAC lymphocytes was somewhat lower; controls, 12.9 ± 0.7%, PBB-exposed, 14.3 ± 0.5%, and PBB greater than 3 ppm/g fat, 12.5 ± 0.8%. Surface Ig-bearing lymphocytes were more abundant than either E or EAC lymphocytes. Control cattle contained 20.3 ± 0.8%, range 11–30%; PBB-exposed cattle contained 31.2 ± 0.7%, range 13–37%; and PBB greater than 3 ppm/g fat contained 22.9 ± 1.3%, range 13–29%. Some sIg-bearing cells also contained C receptors, however, E lymphocytes contained neither C nor sIg receptors. Erythrocytes from other animals species including rat, mouse, human, and rabbit were incubated with bovine lymphocytes. However, no significant numbers of rosette-forming cells were observed.

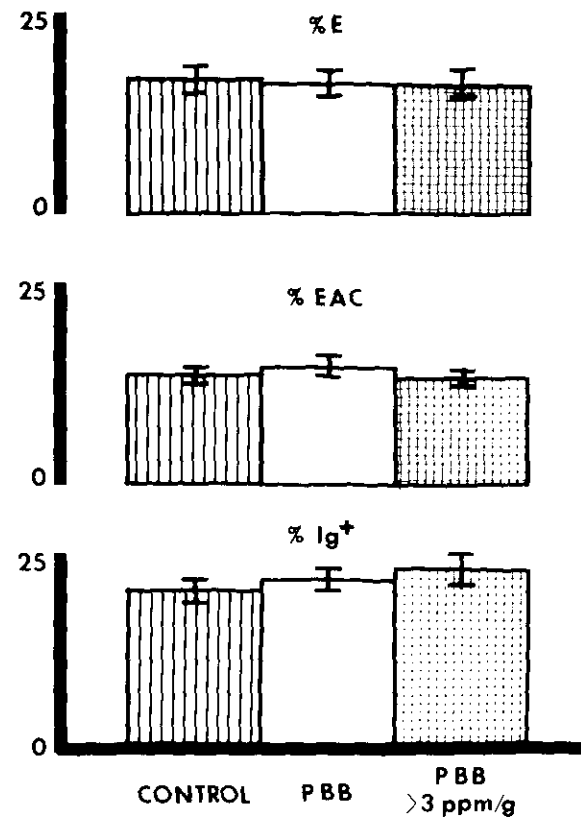

FIGURE 2. Lymphocyte subpopulations in control and PBB-exposed cattle.

Immune Competence

Several tests were employed to evaluate both humoral and cell-mediated immune functions in cattle exposed to PBBs. Lymphocyte blastogenesis studies have become a routine procedure to evaluate the functional status of lymphocytes. DNA synthesis in bovine lymphocytes was evaluated by radioisotope incorporation procedures following mitogen stimulation. Figure 3 depicts the results obtained from control and PBB-exposed animals.

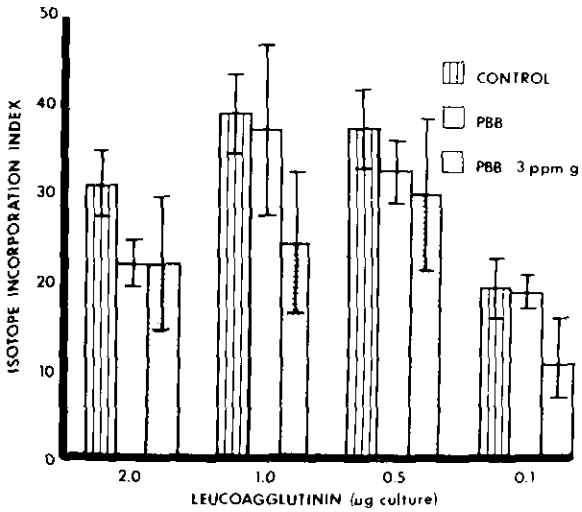

FIGURE 3. Isotope incorporation indexes (III) in lymphocyte cultures from control and PBB-exposed animals stimulated with leukoagglutinin.

The results demonstrate that the mean III values were lower in lymphocyte cultures from cattle exposed to greater than 3 ppm/g fat. However, the values were not statistically different at the 5% level. It must be emphasized that the III within both the control and the PBB groups were quite variable (range, 6–106). Of importance, however, is the observation that each group exhibited a dose response curve and that LA concentration between 0.5 to 1.0 μg/culture appeared to be the optimal stimulating dose. A level of 2 μg LA/culture was less effective in stimulating cells than 1.0 μg LA/culture. Bovine lymphocytes were also stimulated with varying concentrations of Con A and PWM. The results presented in Figure 4 show the blastogenic response of bovine lymphocytes stimulated with the optimal concentrations of these phytolectins. Both Con A and PWM stimulated a significant blastogenic response in all animals studied. No statistically significant difference was observed, even in animals exposed to higher concentrations of PBB.

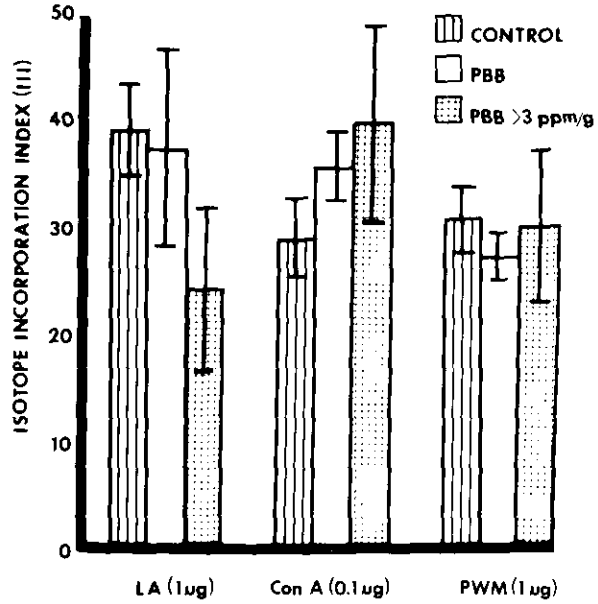

FIGURE 4. Isotope incorporation indexes (III) in lymphocyte cultures from control and PBB-exposed animals stimulated with Leukoagglutinin, Concanavalin A, or Pokeweed Mitogen.

Although the blastogenic response of lymphocytes serves as a model of the antigen recognition phase of the immune response, this test does not provide any information regarding the effector phase of immune reactions. Additional studies were completed to evaluate the humoral and cell-mediated immune responses in control and PBB-exposed animals. Fourteen animals were immunized with KLH and the serum titers determined at weekly intervals. Antibody production to KLH occurred within the first week following injection. The antibody titers ($\log_2$) increased during the second week, and peak responses were observed on day 14 in both animal groups (Table 2). The mean peak titers (MPT) in PBB and control animals were similar. Preliminary studies indicate that the antibody in the primary immune response to KLH is primarily of the IgM class although during the decline of the reaction, IgG antibodies were also detected.

Table 2. Antibody response to Keyhole limpet hemocyanin.

Experimental group	Mean peak titer ($\log_2$)[a]
Control (7)[b]	7.8
PBB (7)[b]	8.7

[a] Titer on day 14.
[b] Number in parenthesis indicates number of cattle studied.

The cell-mediated immune response was investigated by using *in vivo* and *in vitro* procedures. Control and PBB-exposed cattle were immunized with 1–5 × 10^8 BCG organisms. After a 35-day interval, animals were challenged interdermally with PPD and observed during the next 48 hr for a delayed hypersensitivity reaction at the injection site. After 24 hr, both animal groups displayed marked, and comparable responses (Table 3). The *in vitro* response of bovine lymphocytes, both control and PBB-exposed, was tested following the incubation of PPD with 2 × 10^5 lymphocytes for 5 days. The results in Table 4 indicate a marked elevation in the III of lymphocytes from immunized animals stimulated with PPD; however, there was no statistical difference between the reactions of control and PBB-exposed animals.

Table 3. ***In vivo*** **cell-mediated immune response.**

Experimental group	PPD response, cm^2[a]
Control (5)[b]	110.2 ± 15.3
PBB (5)[b]	108.1 ± 26.6

[a] Mean area ± standard error of skin reaction 24 hr following intradermal PPD injection.
[b] Number in parenthesis indicates number of cattle studied.

Table 4. ***In vitro*** **cell-mediated immune response.**

Experimental group	Isotope incorporation index (III)[a]
Control (5)[a]	
Unimmunized	< 2
BCG	14.9 ± 4.7
PBB	
Unimmunized	< 2
BCG	14.6 ± 3.2

[a] III determined on day 5 from *in vitro* studies with lymphocytes from cattle before BCG inoculation or 35 days following BCG *immunization*.
[b] Number in parenthesis indicates number of cattle studied.

Serum Studies

The serum from control and PBB-exposed animals was evaluated for immunoglobulin levels, autoantibodies, and cytotoxic and blastogenic inhibitory factors. Concentrations of immunoglobulins, products of B lymphocytes, are affected by both the physiological and pathological status; thus, a direct measurement of immunoglobulin in the serum and other body fluid can provide a rapid analysis of B cell integrity. Figure 5 depicts the immunoglobulin levels obtained from the 35 control animals and the 52 PBB-exposed animals studied. There was no statistical difference between the serum concentrations of IgG, IgM, or IgA in the PBB animals studied. In addition, serum from PBB-exposed animals was not cytotoxic for bovine or human lymphocytes incubated either in the presence or absence of a biologically active rabbit complement (data not shown). Further, incubation of serum from PBB-exposed animals (25% v/v) either with autologous or allogeneic lymphocytes did not impair the blastogenic response of these cells to 1 μg LA/culture. A representative example is shown in Table 5. Serum from PBB-exposed animals were also tested for autoantibodies including antinuclear, mitochondrial and smooth muscle antibodies. There was no evidence in any sera studied from 15 animals with PBB levels greater than 1 ppm/g fat for autoantibodies (data not shown).

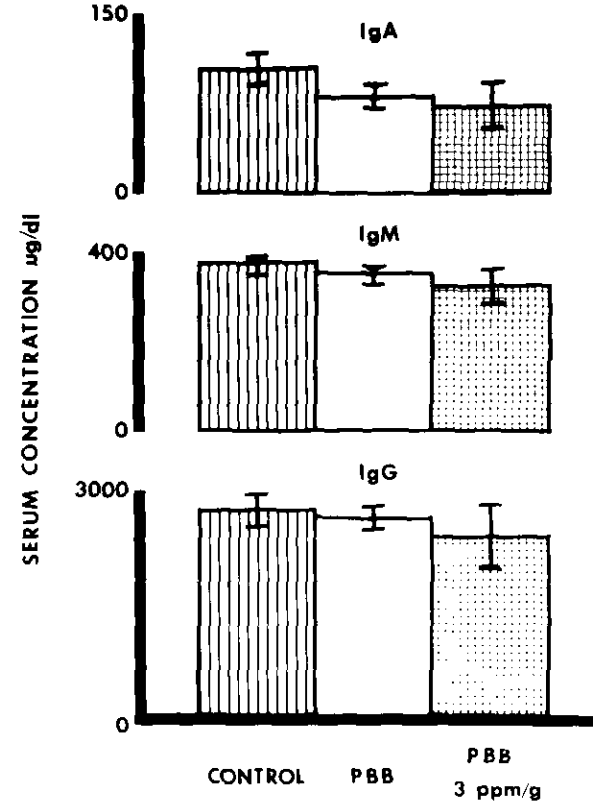

FIGURE 5. Serum immunoglobulin concentrations in control and PBB-exposed animals.

Discussion

The immune system functions to protect the complex biochemical integrity of the host by preventing the invasion of foreign, biologically active materials which may cause malfunction of highly specialized tissue. The rapid elimination of bacteria,

Table 5. Serum effects on lymphocyte blastogensis.

Serum Source[a]	Isotope incorporation index (III)[b]
Fetal calf serum	12.4
Autologous serum	5.9
# 1608	14.6
# 1609	23.3
# 1610	7.7
# 1611	8.4
# 1612	19.1
# 1614	10.4
# 1615	29.3

[a] Serum was used at 25% v/v concentration; fetal calf serum was obtained from Reheis, Chicago, Ill.; serum from 1608-1615 were obtained from cattle at the Ohio Agricultural Research and Development Center. PBB values ranged up to 30 ppm/g fat.

[b] III determined on day 3 in cultures stimulated with 1.0 μg LA/culture.

viruses, toxic substances, and perhaps spontaneously arising neoplastic cells is essential for the survival of multicellular organisms. The mammalian immune system has evolved sophisticated mechanisms which allow the recognition of self from non-self. Recognition of foreign substances would be biologically unimportant, however, unless processes were also available for the localization and destruction of foreign substances. The specific mechanisms for localization and destruction of foreign agents have evolved and these include the production of specifically reactive proteins (antibodies) and the production of specifically modified reactive cells (sensitized lymphocytes).

Recent developments in experimental immunology have advanced our understanding of the morphological heterogeneity of cells participating in immune responses and the selective participation of B lymphocytes in antibody production and that of T lymphocytes and cell-mediated immune reactions. *In vitro* and *in vivo* test systems are now available to enumerate lymphocyte subpopulations and to quantitate humoral and cell-mediated immune events.

To determine the influence of an agent on immune competence, quantitation of lymphocyte subpopulations and effector events are required. In the present study, PBB concentrations up to approximately 30 ppm/g fat did not alter the percentage of lymphocytes with receptors for E, EAC or sIg. Likewise, hematological parameters, WBC and RBC counts, hemoglobin content and hematocrit or the blood differential profile was not altered in cattle contaminated with PBB.

Further studies to evaluate lymphocyte functions revealed that DNA synthesis in lymphocytes from cattle exposed to PBB greater than 3 ppm/g fat was somewhat lower, but not statistically significant when compared to control animals. However, lymphocytes from the same cattle were equally responsive or even more responsive than lymphocytes from control animals when Con A or PWM were used as stimulants. The lymphocyte blastogenic response provides useful information only when there is little or no DNA synthesis in lymphocyte cultures. Values from normal animals regardless of species have varied widely and thus most laboratories have established a cutoff value, below which values from control animals do not occur. Similarly, most immunologists consider a lymphocyte blastogenic response abnormal when there is little or no DNA synthesis in lymphocyte cultures that are stimulated with at least three different photolectins. Finally, lymphocyte blastogenic responses can only be considered abnormal when there is little or no DNA synthesis following incubation with different mitogenic agents studied over a wide range of concentrations. Several studies have documented individual variability to mitogen concentrations and thus an optimal dose for an entire animal species does not exist.

The effector phase of the immune response was studied following immunization of control and PBB-exposed cattle with KLH or BCG. Antibody production to KLH was similar in control and PBB-exposed animals. Likewise, the cell-mediated response, manifested either by a skin reaction to PPD *in vivo* or the incorporation of tritiated thymidine following *in vitro* PPD immunization of lymphocytes from BCG-stimulated animals, was similar in both groups. The elicitation of antibody production and delayed hypersensitivity responses requires coordinated cellular interactions and metabolic processes. That these events were not compromised in PBB-exposed animals suggests these cattle are as equally immunocompetent as controls. Additional support for the integrity of the immune system in PBB-exposed animals was the normal value for the serum immunoglobulin concentrations. Moreover, serum from PBB-exposed cattle was not lymphotoxic, did not impair the blastogenic response of bovine lymphocytes, and contained no autoantibodies to nuclear, mitochondrial or smooth muscle antigen. These autoantibodies have often been detected in humans with liver pathology. However, since Moorhead et al. (*12*) did not observe any remarkable liver damage at necropsy for animals with similar body burdens of PBB as those studied in this investigation, the absence of autoantibody was not unexpected.

Other immunological studies with dogs and humans have also been undertaken. In a histopathological approach, Farber et al. (*16*) demonstrated hypocellular lymph nodes in dogs fed toxic doses of PBBs, but not for those given nontoxic

PBB concentrations. The T-lymphocyte-dependent areas were particularly depleted. This observation is not too surprising, since the study by Moorhead et al. (*12*) demonstrated thymus atrophy in animals exhibiting PBB toxicosis. Studies to evaluate immunoglobulin concentrations, circulating lymphocyte populations, antibody formation and cell-mediated immune activities in dogs fed PBBs have not been reported as yet. In the human studies, Bekesi, and co-workers (*17*) have indicated that some farm family members with PBB exposure exhibited abnormally low levels of circulating T and B lymphocytes (and thus elevated percentages of "null" lymphocytes) and a diminished lymphocyte blastogenic response to PHA and PWM. Unfortunately, these investigators have not indicated the correlation, if any, between those individuals with reduced T or B lymphocyte levels and those with diminished blastogenic responses. Also, it is noteworthy that these authors reported no abnormalities in the hematological profile or the differential cell count in the PBB-exposed population. Further, the serum immunoglobulin concentrations were not altered in the two groups. Important to the interpretation of this data is the fact that (1) these abnormal immune parameters were not PBB-dose related; (2) the control population (Wisconsin farm families) were studied two months after the PBB-exposed group; and (3) the specimens were drawn on site and transported to New York with a delay of 12–18 hr before analysis. Follow-up immunological studies and evaluation by an independent immunology laboratory should be undertaken since the interpretation of values as abnormal, especially when human health is questioned, must be inviolable and conclusive.

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