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Babesia bigemina: Immune Response of Cattle Inoculated with Irradiated Parasites[1]

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BISHOP, J. P., AND ADAMS, L. G. 1974. *Babesia bigemina:* Immune response of cattle inoculated with irradiated parasites. *Experimental Parasitology* 35, 35–43. Effects of various radiation dosages on the infectivity and immunogenicity of *Babesia bigemina* were studied. Calves infected with 1×10^{10} *B. bigemina* parasitized erythrocytes exposed to 24 krad developed progressive parasitemias. Some calves receiving 1×10^{10} parasitized erythrocytes irradiated at 36 krad did not develop progressive infections. Progressive infections were prevented by exposure to irradiation at 48 and 60 krad. A degree of acquired resistance to infection with *B. bigemina* developed in calves after inoculation with parasites irradiated at 48 and 60 krad. The resistance developed was sufficient to suppress multiplication of the *Babesia* and to permit calves to survive otherwise severe clinical infections due to challenge with nonirradiated parasites. Irradiated parasites were frozen without apparent loss of immunizing properties.

INDEX DESCRIPTORS: Cattle; Babesiosis; *Babesia bigemina;* Irradiation; Attenuation; Immunity; Serology; Hematology; Growth.

INTRODUCTION

Bovine babesiosis is a tick-borne hemaprotozoan disease which occurs in the warmer areas of all continents. Attempts to produce immunity against bovine babesiosis have been undertaken by several investigators with varying degrees of success. Cattle can be artificially immunized against bovine babesiosis by injecting infected blood followed by chemotherapy in animals which show clinical signs. Although cattle infected in this way are relatively immune to natural infections, they carry prolonged latent infections and may serve as reservoirs of the parasite for continued tick dissemination of the disease.

Cattle can be partially protected against homologous challenge by immunization with killed *Babesia;* however, large amounts of antigen are required to produce only a marginal protection. The inconclusive results obtained with killed vaccines may be attributed to the loss or denaturation of functional antigens during the preparation of the vaccines or to the rapid elimination of parasites in the host. Therefore, a major problem confronting researchers is how to produce a strong protective immunity without the pathogenic effects resulting from replicating *Babesia.*

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Irradiation of protozoa interferes with their physiologic processes and frequently inhibits their normal development and multiplication (Giese 1967). Studies on the effects of ionizing radiations on parasitic protozoa have repeatedly shown that while an extremely high radiation dose is necessary to cause immediate death of the parasite, much lower doses can interfere with infectivity (Kimball 1955). The immunogenicity of irradiated *Babesia rodhaini* (Phillips 1970, 1971), *Plasmodium spp.* (Corradetti, Verolini and Bucci 1968; Nussenzweig, Vanderberg and Most 1969; Sadun, Wellde and Hickman 1969) and *Trypanosoma spp.* (Duxbury and Sadun 1969, 1970; Sanders and Wallace 1966) in experimental animals (mice, rats, and owl monkeys) has been investigated. Parasites irradiated with a dose which abolished their reproductive potential and ability to produce patent infections may retain their capacity to produce an immune response in a susceptible experimental animal. Therefore, the use of irradiated *Babesia* as vaccines might provide the special immunological properties of living protozoa while suppressing the pathogenic effects.

The following series of investigations were undertaken to study the effect of various radiation dosages on the infectivity and immunogenicity of erythrocytic stages of *Babesia bigemina*. The effect of freezing on the immunogenicity of irradiated *B. bigemina* was also studied. The studies were conducted toward the ultimate goal of producing irradiated vaccines against babesiosis.

Materials and Methods

Experimental Animals

Three-day-old male Holstein calves were obtained from near Facatativá, Colombia and were hand-raised to 3 months of age on the Tibaitatá Instituto Colombiano Agropecuario (ICA) experiment station, in an area free of *Boophilus microplus*. The calves were housed for the duration of the experiment in a tick-free environment at the ICA Laboratorio de Investigaciones Médicas Veterinarias (LIMV) in Bogota, Colombia.

Preparation of Irradiated and Challenge Inocula

Blood containing *B. bigemina* parasitized erythrocytes was collected from splenectomized calves during the acute stage of babesiosis after inoculation with 50 ml of stabilate of *B. bigemina*. The isolation, separation and preservation of the *B. bigemina* stabilate used throughout this study has been previously described (Bishop *et al.* 1973). Although calves were inoculated and challenged at different times, each inoculum was prepared from splenectomized calves previously inoculated with 50 ml of the same stabiliate of *B. bigemina*.

For irradiation treatment, 120 ml of inoculum was divided equally between 2 plastic petri dishes, 1.3 cm deep and 9.1 cm in diameter. The depth of inoculum in each petri dish was approximately 1.0 cm. The inoculum in one petri dish was exposed to the desired amount of radiation using a 3129-c cobalt-60 teletherapy unit (Eldorado 8, Atomic Energy of Canada Limited: Commercial Products, P.O. Box 93, Ottawa, Canada) which delivered a dose rate of approximately 200 rads/min at an exposure distance of 50 cm. The dose rate was determined by ferrous chemical dosimetry and periodic verification of the dose rate was carried out by means of a dosimeter (Victoreen r-Meter Mod. No. 570. Victoreen Instrument Division, 10101 Woodland Ave., Cleveland, OH 44104). The inoculum in the other petri dish was placed in an adjacent room for the duration of the irradiation and used as a nonirradiated control.

Experimental Procedures

Four experimental groups of 4 calves each were given 1 intravenous inoculation of blood containing 1×10^{10} *B. bigemina*

parasitized erythrocytes previously exposed to radiation doses of 24, 36, 48 and 60 krad. A fifth experimental group of 4 calves was given an intravenous inoculation of blood containing 1×10^{10} *B. bigemina* parasitized erythrocytes previously exposed to 60 krad and frozen (Bishop *et al.* 1973) for 48 hr. All calves in the 5 experimental groups were challenged intravenously with blood containing 1×10^{10} *B. bigemina* parasitized erythrocytes 4 weeks after inoculation.

The first control group of 3 calves was given an intravenous inoculation of nonirradiated blood containing 1×10^{10} *B. bigemina* parasitized erythrocytes. To evaluate the possibility that some reduction in parasitemia might be the result of reduction in the number of viable parasites injected rather than an attenuation of the parasites injected, the second and third control groups of 3 calves each received an intravenous inoculation of nonirradiated blood containing 1×10^{7} and 1×10^{4} *B. bigemina* parasitized erythrocytes, respectively. To test the hypothesis that living erythrocytic stages of *B. bigemina* exposed to a radiation dose sufficient to prevent progressive parasitemias are more immunogenic than nonliving parasites, the fourth control group of 3 calves was given an intravenous inoculation of blood containing 1×10^{10} *B. bigemina* parasitized erythrocytes previously heat inactivated in a water bath at 56 C for 30 min. The fifth control group of 3 calves received an intravenous inoculation of a similar volume of nonparasitized irradiated blood. All calves in the 5 control groups were challenged intravenously with blood containing 1×10^{10} *B. bigemina* parasitized erythrocytes 4 weeks after inoculation.

Assessment of Reactions

The calves were examined daily for the presence of parasitized erythrocytes by the use of combination thin and thick blood films. The method used for the preparation of the combination thin and thick films has been previously described (Bishop and Adams 1973).

Subinoculations were performed the day of challenge from groups which failed to demonstrate detectable parasitemias to determine whether subpatent parasitemia had resulted. Subinoculations were accomplished by collecting 100 ml of blood from each calf in each group by venipuncture and injecting the pooled blood from each group into splenectomized calves.

Daily packed cell volumes were determined by the microhematocrit method and rectal temperatures were measured each morning throughout the experiment. All calves were weighed weekly following a 12-hr withdrawal from feed.

Determination of babesial complement fixing (CF) serum antibody levels was performed twice a week by a microtiter procedure similar to that described by Hidalgo and Dimopoullos (1967). *Babesia bigemina* antigens for the CF microtiter procedure were prepared from blood containing 23% *B. bigemina* parasitized erythrocytes by a method described by Mahoney (1967). Antigens were titrated against a group of 8 positive reference serums according to a scheme previously described (Anonymous 1958). The positive serums were taken from cattle 1 to 4 months following recovery from blood-induced infections. A unit of antigen was defined as the highest dilution causing complete complement fixation in the presence of the weakest positive reference serum diluted 1:5.

Results

The results summarized in Table 1 show that calves inoculated with 1×10^{10} *B. bigemina* parasitized erythrocytes exposed to 24 krad developed progressive parasitemias with prolonged prepatent periods and lower maximum parasitemias than control calves inoculated with 1×10^{7} nonirradiated parasitized erythrocytes. Three of 4 calves receiving parasitized blood irradiated at 36 krad did not develop progressive

TABLE I

Characteristics of B. bigemina *Infections Produced with Infected Blood Subjected to Different Radiation Doses*

Radiation dose of inoculum (krad)	No. of parasitized erythrocytes inoculated	Reaction after inoculation of irradiated *B. bigemina* parasitized erythrocytes[b]							
		No. calves with parasitemia/no. calves inoculated[a]	Prepatent period (days)	Maximum % parasitized erythrocytes	Maximum temperature (C)	Minimum packed cell vol (%)	Maximum complement fixation titer	Avg daily gain (g/day)	Deaths
0 (controls)	1×10^{6}	3/3	2.0(2–2)[c]	3.2(0.4–8.0)	41.5(41.3–41.6)	18(13–28)	1:320(1:160–1:640)	423(71–821)	0
0 (controls)	1×10^{6}	3/3	5.7(5–6)	0.03(0.008–0.06)	40.5(40.3–40.7)	27(24–32)	1:201(1:160–1:320)	798(643–911)	0
0 (controls)	1×10^{6}	0/3	None	None	39.7(39.2–40.0)	27(24–28)	Neg. (Neg.–Neg.)	863(643–1036)	0
24	1×10^{6}	4/4	6.5(6–8)	0.11(0.008–0.40)	40.3(39.5–40.7)	26(22–30)	1:135(1:80–1:320)	661(500–786)	0
36	1×10^{6}	1/4	13.0(13–13)	0.002(0.002–0.002)	40.1(39.7–40.3)	30(26–33)	1:13(Neg.–1:80)	790(536–1000)	0
48	1×10^{6}	0/4	None	None	40.1(39.6–41.0)	31(29–34)	1:2(Neg.–1:5)	875(661–982)	0
60	1×10^{6}	0/4	None	None	40.6(40.0–40.9)	30(28–31)	1:3(Neg.–1:20)	857(786–1018)	0

[a] Characteristics of reactions based on data collected for 28 days postinoculation.

[b] Detected by either thick blood films or subinoculations.

[c] Range of means.

TABLE II

Immune Response of Calves Inoculated Once with Irradiated B. bigemia ***and Challenged 4 Weeks Later with Nonirradiated*** B. bigemina

Treatment of inoculum (krad)	Size of inoculum	No. calves with parasitemia/no. calves challenged	Prepatent period (days)	Reaction after challenge with 1×10^{10} unirradiated *B. bigemina* parasitized erythrocytes[a]					
				Maximum % parasitized erythrocytes	Maximum temperature (C)	Minimum packed cell vol (%)	Maximum complement fixation titer	Avg daily gain (g/day)	Deaths
0 (control)	1×10^{10} *B. bigemina*	3/3	1.0(1–1)[b]	0.006(0.001–0.008)	40.2(39.9–40.5)	29(28–31)	1:201(1:80–1:320)	887(786–964)	0
0 (control)	1×10^{10} *B. bigemina*	3/3	1.7(1–3)	0.004(0.002–0.008)	40.2(40.1–40.3)	28(28–30)	1:127(1:80–1:160)	976(893–1036)	0
0 (control)	1×10^{10} *B. bigemina*	2/2	1.0(1–1)	0.5(0.5–0.5)	41.3(40.9–41.7)	11(10–12)	1:160(1:160–1:160)	813(696–929)	1[c]
Heat inact. (control)	1×10^{10} *B. bigemina*	3/3	1.0(1–1)	0.8(0.4–1.0)	41.1(39.8–41.8)	19(16–22)	1:640(1:640–1:640)	815(482–1107)	0
36 (control)	10 ml normal blood	3/3	1.0(1–1)	0.6(0.1–1.0)	41.4(41.0–41.7)	10(3–14)	1:227(1:160–1:320)	982(786–1179)	1[d]
24	1×10^{10} *B. bigemina*	4/4	1.0(1–1)	0.003(0.002–0.005)	39.7(39.6–40.0)	27(25–30)	1:134(1:180–1:160)	1093(1000–1264)	0
36	1×10^{10} *B. bigemina*	3/3	2.0(1–4)	0.02(0.008–0.04)	40.0(39.4–40.5)	26(26–27)	1:201(1:160–1:320)	1060(839–1214)	1[c]
48	1×10^{10} *B. bigemina*	4/4	1.0(1–1)	0.1(0.01–0.3)	39.9(39.6–40.4)	24(21–27)	1:380(1:320–1:640)	947(875–1036)	0
60	1×10^{10} *B. bigemina*	4/4	1.5(1–2)	0.03(0.01–0.06)	39.9(39.7–40.1)	25(23–27)	1:269(1:160–1:640)	938(750–1143)	0
60 (frozen)	1×10^{10} *B. bigemina*	2/2	1.0(1–1)	0.06(0.05–0.07)	40.0(40.0–40.0)	23(21–25)	1:640(1:640–1:640)	822(768–875)	1[c]

[a] Characteristics of reactions based on data collected for 28 days postchallenge.
[b] Range of Means.
[c] Death due to acute anaphylaxis following challenge.
[d] Death due to acute babesiosis following challenge.

parasitemias. Progressive infections were prevented by exposure to 48 and 60 krad and subinoculations into susceptible splenectomized calves failed to produce active infections. Control calves that had been inoculated with 1×10^7 nonirradiated parasitized erythrocytes also had prolonged prepatent periods and lower maximum parasitemias when compared to control calves inoculated with 1×10^{10} parasitized erythrocytes.

The results summarized in Table II show that all calves developed progressive parasitemias with similar prepatent periods following challenge with 1×10^{10} nonirradiated *B. bigemina* parasitized erythrocytes. The average maximum parasitemias were 0.8 and 0.6% following challenge for calves inoculated 4 weeks previously with 1×10^{10} heat inactivated parasitized erythrocytes and 10 ml nonparasitized irradiated blood, respectively. The average maximum parasitemias were 0.03 and 0.06% following challenge for calves inoculated 4 weeks previously with 1×10^{10} parasitized erythrocytes irradiated at 60 and 60 krad (frozen), respectively.

The average maximum morning rectal temperatures were 41.1 and 41.4 C following challenge for calves inoculated 4 weeks previously with 1×10^{10} heat inactivated parasitized erythrocytes and 10 ml nonparasitized irradiated blood, respectively. The average maximum morning rectal temperatures were 39.9 and 40.0 C following challenge for calves inoculated 4 weeks previously with 1×10^{10} parasitized erythrocytes irradiated at 60 and 60 krad (frozen), respectively.

The average minimum packed cell volumes were 19 and 10% following challenge for calves inoculated 4 weeks previously with 1×10^{10} heat inactivated parasitized erythrocytes and 10 ml nonparasitized irradiated blood, respectively. The average minimum packed cell volumes were 25 and 23% following challenge for calves inoculated 4 weeks previously with 1×10^{10} parasitized erythrocytes at 60 and 60 krad (frozen), respectively.

Three calves (one previously inoculated with 1×10^{10} parasitized erythrocytes irradiated at 24 krad, one previously inoculated with 1×10^{10} parasitized erythrocytes irradiated at 60 krad and one previously inoculated with 10 ml nonparasitized irradiated blood) suffered intense respiratory distress for 15 min following intravenous challenge. Three other calves (one previously inoculated with 1×10^{10} parasitized erythrocytes irradiated at 36 krad, one previously inoculated with 1×10^{4} nonirradiated parasitized erythrocytes and one previously inoculated with 1×10^{10} parasitized erythrocytes irradiated at 60 krad and frozen) suffered intense respiratory distress following intravenous challenge. Coughing produced cream-colored frothy fluids mixed with blood. These calves died within 24 hr following challenge. Postmortem examination revealed lesions which were confined largely to the respiratory system, which included severe intra-alveolar and interstitial edema and emphysema with intra-alveolar hemorrhage of the lung. The trachea and major bronchi contained a hemorrhagic cream-colored frothy fluid. One other calf died following the development of a severe systemic toxic reaction after intravenous inoculation with *B. bigemina* parasitized blood previously exposed to 60 krad and frozen.

Discussion

The results from studies designed to determine the effect of various radiation dosages on the infectivity of *B. bigemina* indicate that calves infected with *B. bigemina* parasitized erythrocytes exposed to 24 krad developed progressive parasitemias whereas progressive infections were prevented by exposure to irradiation at 48 krad. These results confirm and extend those reported recently from cattle inoculated with irradiated *B. bigemina* (Castro and Canabez 1968), *Babesia major* (Brocklesby, Purnell

and Sellwood 1972) and from mice and rats inoculated with irradiated *B. rodhaini* (Phillips 1970, 1971).

The results also showed that in addition to a lower infection rate brought about by irradiated parasites, calves that did become infected had prolonged prepatent periods and lower maximum parasitemias than control calves receiving nonirradiated inocula. The results could be due to the death or developmental arrest of some of the parasites, or to an overall retarded development leading to more slowly developing forms. Control calves that had been inoculated with 1×10^5 nonirradiated parasitized erythrocytes also had prolonged prepatent periods and lower maximum parasitemias when compared to control calves inoculated with 1×10^{6} nonirradiated parasitized erythrocytes. Therefore, the prolonged prepatent periods and lower maximum parasitemias in calves that had received irradiated parasites could have been due in part to a reduction in the number of viable parasites injected.

The finding that calves inoculated with 1×10^5 nonirradiated parasitized erythrocytes had prolonged prepatent periods and lower maximum parasitemias (Table 1) indicate that such a standardized inoculum might be of use as a vaccine to produce an attenuated infection. Callow and Mellors (1966) have developed a similar monovalent vaccine for *B. argentina* prepared in splenectomized calves. Kennon *et al.* (1964) and Pipano (1969) have also reported that the length of incubation period was inversely related and the severity of the reaction directly related to the number of parasites in the inoculum.

A degree of acquired resistance to infection with *B. bigemina* developed in calves after inoculation with *B. bigemina* parasitized blood irradiated at 48 and 60 krad. The resistance developed was sufficient to suppress multiplication of the *Babesia* and to permit calves to survive otherwise severe clinical infections with nonirradiated parasites. There was also less erythrocytic destruction and a smaller increase in rectal temperatures following challenge for calves previously inoculated with *B. bigemina* parasitized blood irradiated at 48 and 60 krad. The irradiated parasites were apparently responsible for the development of this resistance since irradiated nonparasitized blood did not produce a discernible acquired resistance to *B. bigemina*. These results are in agreement with and extend those recently reported from mice and rats immunized with irradiated *B. rodhaini* (Phillips 1970, 1971).

The failure of the immunized calves to develop patent infections following inoculation of irradiated parasites indicate that immunity could not be considered to have been developed in response to a long standing chronic blood infection. The observation that inoculation of calves with irradiated nonreplicating *B. bigemina* may induce protective immunity, suggests that the presence of replicating *Babesia* in the host is not necessary for the development of acquired resistance. This is in agreement with the concept of "sterile immunity" described for *B. argentina* (Callow 1968), *B. bigemina* (Callow 1964, 1967), *B. divergens* (Joyner and Davies 1967), *B. microti* (Cox and Young 1969) and *B. rodhaini* (Cox and Young 1969, Phillips 1969, 1971).

The acquired resistance to infection with *B. bigemina* developed in calves inoculated with 1×10^{6} *B. bigemina* irradiated at 48 and 60 krad was much greater than the acquired resistance to infection developed in calves inoculated with 1×10^{6} heat killed *B. bigemina*. Thus it seems likely that immunization with irradiated nonreplicating *Babesia* may provide the special immunological properties of living parasites important for producing a strong immunity while suppressing the pathogenic effects of the parasite.

Although CF antibodies are probably not functional in immunity (Mahoney 1967a), it was of interest for theoretical and diag-

nostic considerations to determine whether detectable CF antibodies were produced in calves after one inoculation with *B. bigemina* parasitized blood irradiated at 48 and 60 krad. The results of the CF microtiter tests indicated that 3 of 8 calves were able to produce detectable levels of antibodies with maximum titers ranging from 1:5 to 1:20 even when the normal development and multiplication of *Babesia* were interrupted by irradiation. There was no discernible difference in acquired resistance to infection between those calves which did or did not develop detectable CF antibodies.

The success obtained in immunizing calves with *B. bigemina* which had been irradiated, frozen and stored in a dry ice cabinet may be of practical significance in future studies. The *Babesia* parasites could be irradiated and frozen, without apparent loss of immunizing properties, for use in distant parts of the world.

It is of interest to note a consistent temperature rise in calves following intravenous inoculations with *B. bigemina* even when the normal development and multiplication of *Babesia* were interrupted by irradiation or heat inactivation. Vaccines which contain foreign protein can act as pyrogens that cause body temperature rise following injection (Guyton 1961). It is also possible that plasma proteins and other proteins in blood which are not normally pyrogenic or toxic could be changed chemically by hemolysis, degradation, or denaturization and thereafter cause systemic reactions (Guyton 1961). One calf died following the development of a severe systemic toxic reaction after intravenous inoculation with previously frozen *B. bigemina* parasitized blood exposed to 60 krad.

Six calves also developed severe systemic reactions following intravenous challenge with blood containing $1 \cdot 10^{7}$ nonirradiated *B. bigemina* parasitized blood. Aitken and Sanford (1969) have recently described similar anaphylactic reactions in calves given 1 intravenous injection of a foreign protein following challenge by the same route after a latent period of at least 7 days. Anaphylactic shock of this type, however, usually only follows the intravenous injection of the challenge dose of antigen and could probably be avoided by using other routes in which the antigen would reach the circulation more slowly.

Standardized infecting and immunizing doses in the present study were given intravenously because previous studies have indicated that *Babesia* may be more immunogenic when given by the intravenous route (Mahoney 1967b). The same standardized inocula were given intravenously for challenge since the infections produced by these inocula given by the intravenous route had already been characterized. In view of the above observations, a recharacterization of challenge inoculum using a subcutaneous route of inoculation would be warranted for future studies using infected blood for challenge. Experimental or natural field tick challenge would also most likely avoid the problem of anaphylactic reactions following challenge in calves previously inoculated with irradiated *B. bigemina* parasitized blood.

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