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IMMUNITY TO BABESIA IN MICE
I. ADOPTIVE TRANSFER OF IMMUNITY TO BABESIA RODHAINI WITH IMMUNE SPLEEN CELLS AND THE EFFECT OF IRRADIATION ON THE PROTECTION OF IMMUNE MICE

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ABSTRACT

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Immunisation of Balb/c mice against Babesia rodhaini by an amicarbalide-controlled infection resulted in a solid immunity which lasted for 216 days. With spleen cells of immune mice protection could be transferred both to naive mice pretreated with cyclophosphamide. Treatment of naive mice with cyclophosphamide (300 mg/kg) five days before a lethal B.rodhaini inoculation resulted in over 50% survival. This protective effect of cyclophosphamide is explained by its inhibiting effect on suppressor T-cells.

The protection against B.rodhaini challenge infection afforded to immune Balb/c mice was completely resistant to a sublethal irradiation of 400 rad. Since B-lymphocyte function in antibody production is suppressed by this dose, the role of antibodies in the effector phase of the immunity appears to be of minor if any importance.

A considerable degree of protection was still preserved after irradiation of immune animals with 875 rad. Sensitivity to this irradiation dose of all immunocompetent cells except macrophages and a small fraction of T-lymphocytes indicates the involvement of these cell types in the effector phase of the specific immunity. Highly radioresistant macrophages are therefore considered to play the major role but T-lymphocytes are also required for complete protection.

INTRODUCTION

Successful attempts have been made to convey protection against Babesia to susceptible animals by transfer of spleen cells of immune donor animals. By this method naive rats could be protected against B.rodhaini (Roberts, 1968) and mice against B.microti (Clark, 1976, Ruebush and Hanson, 1980a). The infections in the

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parasite-host models used by these authors ran a benign course in naive animals and seldom ended fatally. Reported here are results of experiments with mice to transfer immunity against a highly pathogenic B.rodhaini strain with spleen cells. The experiments involved the initial production of donor mice with a solid sterile immunity.

The lymphocyte seems to be the major cell responsible for the immunologic memory. Ruebush and Hanson (1980a) have shown that the protective activity of spleen cells of mice immune to B.microti was abrogated by treatment with anti-θ-serum but not with anti-immunoglobulin serum. The role of T-lymphocytes for the induction of immunity against Babesia is further confirmed by Clark and Allison (1974) who observed an increased susceptibility of athymic nude mice to B.microti. However, the protective mechanism of this T-cell dependent immunity is unknown.

Protection of mice against Babesia is also obtained non-specifically by pretreatment with Bacillus Calmette-Guerin (BCG) (Clark et al, 1976) and by a variety of micro-organisms and substances with macrophage activating and/or interferon inducing potentials (Clark, 1979a). It has been suggested that the final effector mechanism may be the same in both non-specific and specific protection (Clark et al., 1977a,b). In each case protection is equally solid and durable, and leads to intra-erythrocytic death of the babesial organisms with very similar ultrastructural changes. The similarity in the heterologous protection of mice against a variety of Babesia and Plasmodium species following both B.rodhaini immunization (Zivkovic et al., 1983) and BCG treatment (Clark et al., 1976), may also be an indication of a comparable effector mechanism.

A remarkable characteristic of the non-specifically induced protection against Babesia is its resistance to irradiation. Exposure of mice treated with BCG (Clark et al., 1977c) or Coxiella burnetii extract (Clark, 1979b) 4 weeks previously to total body irradiation (900 rad) did not disturb their protection against B.microti infection. This article reports results of experiments on the radiosensitivity of the protection against B.rodhaini in mice induced by specific immunization in order to obtain further evidence for a common final effector mechanism in both non-specific and specific protection.

MATERIAL AND METHODS

Experimental animals

All mice were of the Balb/c strain, bred at the Department of Tropical Veterinary Medicine and Protozoology of the Faculty of Veterinary Medicine, Utrecht. Animals were 6-8 weeks old when the experiments were initiated. Mostly females were used, but sex did not appear to affect the outcome. Animals were housed in plastic cages at a room temperature of 23 ± 2°C and 50-60% relative humidity. Food* and tap water were constantly available. Animals irradiated lethally (875 rad) received 130 mg streptomycin and 300 mg neomycin sulphate per liter of drinking water.

Protozoa

The Antwerp strain of Babesia rodhaini was used as stabilate stored in liquid nitrogen as a 50% dilution of heavily infected blood (~ 40% parasitaemia) in RPMI containing 8% dimethyl-sulphoxide or as fresh parasitized blood. Intraperitonal (i.p.) inoculation of 0.1 ml of the stabilate diluted 1:10 with RPMI 1640 produced infections which ended fatally in all mice. Fresh parasitized blood for inoculation was obtained after first passage of the stabilate from mice with a rising parasitaemia between 20 and 40%.

The course of parasitaemia was monitored daily or every second day by examining Giemsa-stained thin blood smears. Parasitaemias were expressed as the percentages of erythrocytes infected.

Immunization procedure

To obtain a solid, sterile immunity mice were immunized by a method slightly modified from Cox and Young (1969). In brief: mice were inoculated by i.p. injection with 0.1 ml stabilate, diluted 1:10 with RPMI 1640**. The resulting infection, with a prepatent period of about a week was controlled by a single i.p. injection of amicarbalide 12 mg/kg***. By varying the period between the initial inoculation and drug treatment, the optimal time for amicarbalide administration was estimated. Two weeks after treatment a high challenge-booster inoculation of 1:10 diluted fresh blood was given

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i.p. Two weeks after this booster inoculation the mice were treated with amicarbalide (24 mg/kg i.p.).

To investigate if this drug treatment completely eliminated the remaining parasites, immunized animals were splenectomized and monitored regularly for parasitaemia. The presence of parasites in immunized mice was also tested by subinoculation of blood- and organ homogenates into naive mice. From 5 immunized mice blood was collected by cardiac puncture in heparinized tubes. Liver, spleen, brain and kidneys from these animals were removed aseptically, collected in RPMI 1640 medium and homogenized separately in a Potter-Elvehjem tube. Homologous homogenates were pooled and samples injected i.p. into 3 splenectomized naive mice in an amount of 1 ml per mouse. Recipient mice were monitored for parasitaemia by tail blood smears at 3-day intervals for 4 weeks.

The duration of the immunity was examined by observing the results of heavy challenge inoculations of fresh parasitized blood given 15, 55, 97, 162 and 216 days after the last amicarbalide treatment.

Transfer of protection against B.rodhaini with spleen cells

Spleens were removed from euthanized mice, cut into small pieces which were gently squeezed through nylon netting (225 µm openings) into RPMI 1640. The cells were washed twice by centrifugation, 5 minutes at 120 g in RPMI 1640, counted electronically* and tested for viability by trypan blue dye exclusion. All procedures were carried out aseptically at 0-4°C.

Spleen cells were injected i.p. into recipient mice, treated with 300 mg cyclophosphamide** /kg i.p. 8 hours previously. The aim of this treatment was to improve the homing of the donor cells. Challenge inoculations of 0.1 ml stabilate diluted 1:10 were given i.p., 5 days after cell transfer. Since pre-treatment with cyclophosphamide (CY) modified the course of parasitaemia, spleen cells were also transferred to untreated naive recipient animals.

Irradiation experiments

Irradiation was carried out at the National Institute of Public Health, Bilthoven, The Netherlands (Courtesy of Dr. J.G. Vos),

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* Coulter Counter, Model 2F, Coulter Electronics, Dunstable, Bedfordshire, England.

** Endoxan, Asta, Bielefeld, Germany.

using a ^{60}Co source of γ rays.

Groups of 7 immunized and non-immunized mice were irradiated with 400 rad and challenged with 0.1 ml of the stabilate 1:10 on day 2, 7, 11 or 21 after irradiation.

Groups of 10 immunized and non-immunized mice were irradiated with 875 rad, reconstituted with $1x10^7$ bone-marrow cells from naive animals and challenged with stabilate diluted 1:10 on day 14 after irradiation.

RESULTS

Immunization of mice against Babesia rodhaini

Timing of the first drug treatment. Since the B.rodhaini strain used causes a 100% lethal infection in mice, the moment of the first drug treatment is of great importance to obtain an optimal protection against challenge infections. Optimal protective immunity was obtained when amicarbalide treatment was given on the second day of patency when the parasitaemia was 10-20%. Earlier treatment resulted in poor immunity with mortality from the challenge infection, whereas late treatment could not prevent mortality from the primary infection.

Absence of parasites in mice after second drug treatment. In order to establish the absence of B.rodhaini parasites in immunized mice after a 24 mg amicarbalide/kg treatment, tail blood smears were examined for two months (at 3-day intervals) and always found negative. Five immunized mice which were splenectomized 10 days after the second (amicarbalide 24 mg/kg) treatment were all found negative on examination of tail blood smears. However, from a group of five immunized mice which were not given a second drug treatment 2 animals demonstrated a relapse of the infection after splenectomy and died.

It is possible that parasites are still present after amicarbalide treatment but that their development is effectively inhibited in the immune host. Therefore blood and homogenates from liver, spleen, brain and kidneys were transferred to splenectomized naive animals. None of these recipients developed parasitaemia, as determined by tail blood smears.

Duration of immunity. At various intervals after immunization mice were challenged with high doses of B.rodhaini parasites (1:10 diluted fresh blood) and the course of parasitaemia was monitored. For 216 days after immunization the mice were fully protected. However, at 162 and 216 days after immunization the peak parasitaemias became somewhat higher than at 15, 55 and 97 days after immunization, when the level of parasitaemia always remained below 1%.

Transfer of protection with immune spleen cells

To investigate if immunization-induced protection against B. rodhaini could be transferred with lymphoid spleen cells, recipient mice pretreated with CY 300 mg/kg were challenged with stabilate 5 days after the cell transfer. The results in Table I show that $1x10^8$ spleen cells induced complete protection against challenge infection. After a prepatent period of 6 days 7 animals out of 10 experienced a low grade parasitaemia (less than 1%). The other 3

TABLE I

Transfer of immunity against B.rodhaini in Balb/c mice by spleen cells [a)]

CY 300 mg/kg	Transferred spleen cells	Mortality	Non survivors	Survivors	
			Median survival (days)	Mean patency (days)	Peak parasitaemia (%)
+	1.10^8 immune	0/10	-	5 ± 2	1
+	2.10^7 immune	2/10	12	4 ± 2	1
+	1.10^8 non-immune	6/6	13	-	-
+	none	6/14	13	15 ± 2	20 ± 18
-	none	9/9	14	-	-

a) Recipient mice were pretreated with cyclophosphamide (CY) 300 mg/kg 8 hours before cell transfer. Challenge infections with stabilate were induced 5 days after cell transfer. Parameters of infection are expressed as mean values ± SD.

animals of this group remained negative. At day 15 after challenge parasites were completely cleared from the blood. Recrudescence did not occur during a 60-day observation period. At that time the recipients resisted high dose challenge infections (0.1 ml fresh blood, 30% parasitaemia). Mice which received $2x10^7$ immune spleen cells showed the same course of parasitaemia upon challenge as the recipients of $1x10^8$ spleen cells except for 2 animals which developed parasitaemia comparable to the controls. These 2 animals died at day 12 after challenge. The recipients of $1x10^8$ non-immune spleen cells isolated from naive mice all died with high parasitaemia at a median survival of 13 days. The control mice which did not receive spleen cells nor treatment with CY all died within 14 days after infection. In the control group pretreated with CY all animals developed high grade parasitaemia (> 10%), but 8 out of 14 mice from this group survived. These survivors cleared their parasites less efficiently than the recipients of immune spleen cells. They showed parasites in the blood smears over a 15-day period, whereas the recipients of immune spleen cells displayed significantly shorter patent periods no longer than 5 days (Table I).

Irradiation of B.rodhaini immunized mice

All mice previously immunized against B.rodhaini, irradiated with 400 rad and challenged with stabilate diluted 1:10 at various time intervals after irradiation, showed a low and transient parasitaemia (Table II). In comparison with non-irradiated immunized mice the patent period was slightly prolonged. Otherwise, the course of B.rodhaini infection in irradiated and non-irradiated immunized animals was similar. Mortality did not occur. Thus the protection against B.rodhaini was completely resistant to 400 rad irradiation, irrespective of the interval between challenge and irradiation.

TABLE II

Effect of 400 rad irradiation on Babesia rodhaini infection in immune and non-immune Balb/c mice a)

Radiation	Time interval to challenge (days)	Prepatent period (days)	Peak parasitaemia (%)	Mortality rate	Time of death (days)
		IMMUNE MICE			
+	2	6.3 ± 0.8	0.7 ± 0.5***	0/7	-
+	7	7.4 ± 4.3	4.6 ± 3.2	0/7	-
+	11	8.1 ± 4.9	2.9 ± 1.8	0/7	-
+	21	7.9 ± 1.1	3.0 ± 1.1	0/7	-
-	-	7.5 ± 3.0	3.3 ± 1.3		-
		NON-IMMUNE MICE			
+	2	4.1 ± 0.4	59.2 ±22.7	7/7	10.2 ± 2.5
+	7	3.6 ± 1.0	55.4 ± 8.1	6/7	9.4 ± 1.3
+	11	6.4 ± 1.5**	35.8 ±15.8	6/7	11.8 ± 0.8**
+	21	5.3 ± 1.3*	38.8 ±21.9 b)	4/8	12.5 ± 1.0**
-	-	3.3 ± 1.6	50.5 ±19.4	10/10	9.4 ± 0.5

a) Mice were challenged with Babesia rodhaini stabilate, dilution 1:10. Parameters of infection are expressed as mean values ± SD and the significance of differences with naive controls is indicated *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

b) Peak parasitaemia of survivors was 23.9 ± 11.9.

Although less complete, the protection against B.rodhaini remained present in 875 rad γ-irradiated mice (Table III). From the ten 875 rad irradiated and bone-marrow reconstituted immune mice, eight animals resisted the challenge dose. One animal died 12 days after challenge with a parasitaemia of 26%. The other animal which died 32 days after challenge had a maximum parasitaemia of only 7% nine days previously. This may indicate a cause of death unrelated to B.rodhaini infection. The non-immune irradiated control mice all died between 9 and 15 days after inoculation with a mean peak parasitaemia of more than 60% (Fig. 1). The immune irradiated mice developed a parasitaemia with a mean peak height of 22% (Fig. 1). In comparison with the non-irradiated immune mice this mean parasitaemia was significantly higher and persisted for a prolonged period. However, in all surviving mice the parasites were completely cleared from the blood and recrudescence never occurred (Fig. 1).

TABLE III

Effect of 875 rad irradiation on Babesia rodhaini infection in immune bone-marrow-reconstituted and non-immune Balb/c mice[a)]

Radiation	Time interval to challenge (days)	Prepatent period (days)	Peak parasitaemia (%)	Mortality rate	Time of death (days)
		IMMUNE MICE			
+	14	7.0 ± 3.2	22.0 ± 7.7	2/10	12 and 32
-	-	7.5 ± 3.0	3.3 ± 1.3	0/ 7	-
		NON-IMMUNE MICE			
+	14	5.8 ± 2.0	61.8 ± 18.1	11/11	12.7 ± 1
-	-	3.3 ± 1.6	50.5 ± 19.4	10/10	9.4 ± 0

a) Mice were challenged with B.rodhaini stabilate, dilution 1:10. Parameters of infection are expressed as mean values ± SD.

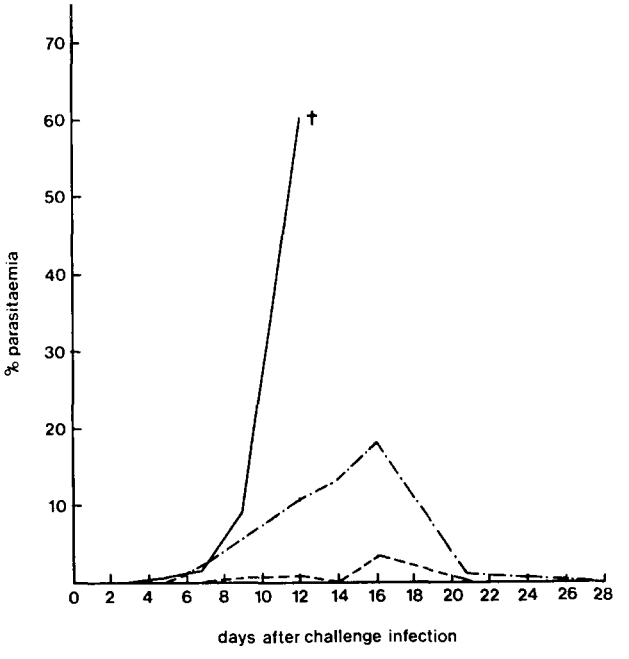

Fig. 1. Effect of 875 rad γ-irradiation on the course of Babesia rodhaini infection in naive mice and mice immunized previously against this parasite.

——— immune mice
------- naive mice
━━━ naive mice, irradiated (875 rad)
—·—·— immune mice, irradiated (875 rad)

Irradiation of non-immunized mice

Naive mice were irradiated with 400 rad and challenged with 1:10 diluted stabilate at various time intervals after irradiation, to serve as controls for the irradiated immune mice.

In these mice high parasitaemias were observed, but some of them survived the challenge infection, in contrast to the non-irradiated naive mice which all died (Table II, Fig. 2). The protection against B.rodhaini was most pronounced when the animals were challenged 21 days after irradiation. At this time interval between irradiation and challenge infection 4 out of 8 animals survived. The non-survivors of this group lived significantly longer than the controls ($P < 0.001$). At an interval of 11 days the survival time was significantly prolonged ($P < 0.001$) as well (Table II). In both groups the prepatent period was also significantly longer.

The course of a B.rodhaini infection in naive mice was not modified by irradiation with 875 rad (Fig. 1, Table III).

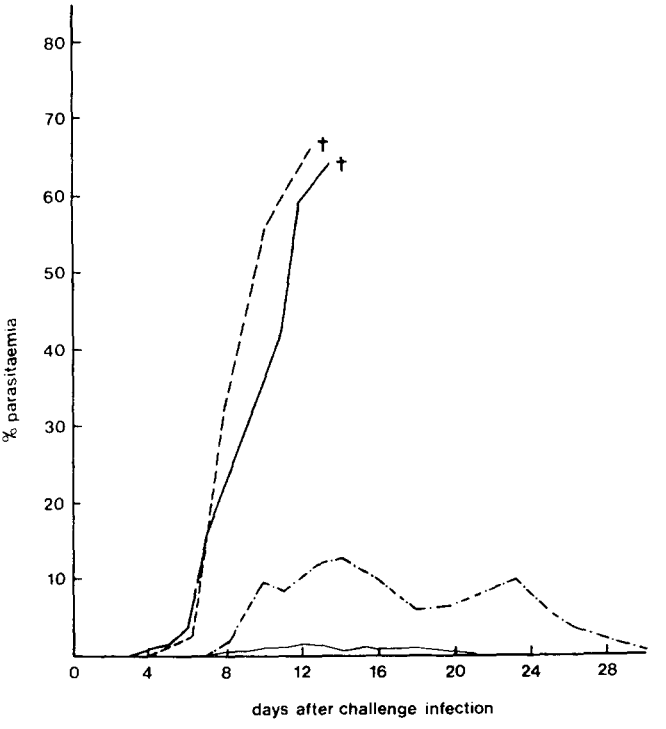

Fig. 2. Course of Babesia rodhaini infection in naive mice exposed to 400 rad γ-rays 21 days previously; (—•—) survivors, (——) non-survivors, (•••) immune mice.

DISCUSSION

With amicarbalide the lethal infection of B.rodhaini in mice can be controlled effectively leaving the surviving animals with a solid immunity (Cox and Young, 1969; Roberts et al., 1972; Mitchell, 1977). The timing of the drug treatment was found to be of great importance for successful immunization.

Splenectomy or subinoculation of blood and organ homogenates failed to demonstrate the presence of parasites in immune animals which had been treated with amicarbalide 24 mg/kg. Mice with such immunity resisted comparable challenge inoculations given up to 216 days after the 24 mg/kg amicarbalide injection.

Since subinoculation of spleen homogenates from immunized mice did not induce infections in naive recipients, these immune animals were suitable as donors in cell transfer experiments. A dose of 1×10^8 immune spleen cells gave good protection against the challenge infections. The protection of 2×10^7 immune spleen cells was less complete.

It is remarkable that 8 mice out of 14 pretreated with CY but not receiving spleen cells survived the challenge infection. This protective effect cannot be explained by a direct effect of CY on the parasite since the drug is rapidly eliminated from the body. The protective effect may be related to a heightened immune response. After a single injection of 300 mg CY/kg, antibody production is completely suppressed (Willers and Sluis, 1975). The cell-mediated immune reactivity is slightly reduced during 4 days after CY treatment, but this reduction is followed by an enhancement, most pronounced at day 7. This enhancement can be eliminated by the administration of spleen cells (Kerckhaert et al., 1974). Mitsuoka et al. (1979) have given strong evidence that the enhancement of cell-mediated immunity by CY is caused by elimination of suppressor T-cells, which are more sensitive to CY than are effector T-cells. Possibly, the different survival rates of the CY-treated mice receiving normal spleen cells and those not receiving cells may be explained by the presence of suppressor cells in the cell inoculum. This suggests that an increased T-cell reactivity may be responsible for the protection of CY-treated mice against B.rodhaini. The observation that congenitally athymic nude

mice could not be immunized against B.rodhaini by an amicarbalide controlled infection (Zivković et al., 1983) further supports the essential role of T-lymphocytes in the induction of immunity against this parasite.

The suppressive effect of ionizing radiation has been known to increase susceptibility to different pathogenic microorganisms but also to impair or completely abolish already established immunity, depending on the dose used. In our experiments it was observed that the established immunity against Babesia rodhaini was completely resistant to a dose of 400 rad and that a considerable degree of protection was preserved after exposure to 875 rad. The 400 rad irradiated immune mice developed the same pattern of parasitaemia upon challenge infections as the non-irradiated immune controls. This radiation dose significantly suppresses B-lymphocyte functions such as antibody formation (Anderson and Warner, 1976) and prevents their participation in the immune response. Thus a B-lymphocyte independent mechanism is involved in the effector phase of the specific immunity. This is in agreement with previous observations that protection against Babesia in mice could not be transferred with hyper-immune serum (Clark, 1976; Roberts et al., 1972; Roberts and Tracey-Patte, 1975). A B-cell independent mechanism was also observed in immunity against Babesia microti in mice by Ruebush and Hanson (1980a) who demonstrated that transfer of immunity by spleen cells was not affected by pretreatment of the cells with anti-immunoglobulin serum.

Although the 875 rad irradiated immune mice were considerably protected against the subsequent challenge infection, some impairment of the immunity was noted as shown by an increased and prolonged parasitaemia. Parasitaemia-related mortality occurred in only one out of ten animals.

From the cells involved in a protective immune response only macrophages (Smith et al., 1967; Muramatsu et al., 1965; Anderson and Warner, 1976) and a small fraction of T-lymphocytes (Kataoka and Sado, 1975) are resistant to 875 rad irradiation. Since treatment with betamethasone (Young and Cox, 1971) enhanced parasitaemia and caused recrudescences in Babesia-infected animals and treatment with dexamethasone (unpublished results) easily abrogated an established immunity to Babesia rodhaini, macrophages, as a corticosteroid-sensitive cell population, are thought to be essential for protection.

Macrophage activation following ionizing irradiation has been reported previously (Cheers and Waller, 1975; Anderson and Warner, 1976). Whether this non-specific macrophage activation contributes to the protection observed in immune irradiated animals from our experiments is unclear. Probably it is responsible for the surprising number of survivors among the non-immune, irradiated (400 rad) animals (Table II, Fig. 2).

The role of T.lymphocytes in the effector phase of the immunity against Babesia is uncertain. After treatment of B.rodhaini immunized with anti-thymocyte serum (unpublished data) the animals resisted a subsequent challenge infection, but this protection was incomplete. Both after anti-thymocyte serum treatment and after 875 rad irradiation, immune mice developed a considerable degree of parasitaemia during a prolonged patent period. These results suggest that T-lymphocytes are important in maintaining complete protection.

A T-lymphocyte-independent protection against Babesia microti and Babesia rodhaini in mice treated with live BCG was originally observed by Clark et al. (1976). Later on protection against Babesia was noted in mice pretreated with a great variety of agents (Clark, 1979a). This non-specifically induced protection is supposed to be related to macrophage activation (Clark, 1979; Allison and Clark, 1977). The resistance to lethal irradiation of the specifically induced anti-Babesia immunity reported here, is comparable with radio-resistance of the non-specific protection induced by BCG (Clark et al., 1977c). This may be an indication of the same final mechanism, involving macrophages, in the specifically and non-specifically induced protective response to Babesia rodhaini. However, in the effector phase of the specifically induced immunity a depletion of T-lymphocytes by 875 rad causes an enhancement of parasitaemia whereas this is not the case in the non-specifically induced immunity. This suggests that T-lymphocytes probably play an important role in the effector phase of the specifically induced immunity to B.rodhaini.

The nature of the protective mechanism underlying parasite clearance during Babesia infection is not known and can only be speculated upon (Allison and Clark, 1977; Clark, 1978).

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