AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D. C. 20523
BIBLIOGRAPHIC INPUT SHEET

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1. SUBJECT CLASSIFICATION
A. PRIMARY: PUBLIC HEALTH
B. SECONDARY: GENERAL PUBLIC HEALTH

2. TITLE AND SUBTITLE
SOME QUALITATIVE ASPECTS OF SPLEEN CELL TRANSFER STUDIES ON PLASMODIUM BERGEI-INFECTED RATS

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4. DOCUMENT DATE: 1973
5. NUMBER OF PAGES: 4 PAGES
6. ARC NUMBER: ARC 614.532- C117a

7. REFERENCE ORGANIZATION NAME AND ADDRESS
UNIVERSITY OF NEW MEXICO
DEPT. OF BIOLOGY
ALBUQUERQUE, NEW MEXICO 87106

8. SUPPLEMENTARY NOTES *(Sponsoring Organization, Publishers, Availability)*

PUBLISHED IN THE JOURNAL OF PROTOZOOLOGY-VOL.20-NO.3

9. ABSTRACT
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10. CONTROL NUMBER: PN-AAB-037
11. PRICE OF DOCUMENT:
12. DESCRIPTORS
ADOPTIVE IMMUNITY IN RATS, PARASITE, CELL MEDIATED IMMUNITY, INFECTION, PARASITEMIA
13. PROJECT NUMBER: 931-17-511-522
14. CONTRACT NUMBER: AID/CSD-3689
15. TYPE OF DOCUMENT: RESEARCH PAPER

AID 590-1 (4-74)

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J. PROTOZOOL., 20(3), 449–452 (1973).
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J. PROTOZOOL. 20(3), 449-452 (1973).

Some Qualitative Aspects of Spleen Cell Transfer Studies on *Plasmodium berghei*-Infected Rats*

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SYNOPSIS. Adoptive immunity to *Plasmodium berghei* was transferred by intraperitoneal injections into rats, never before exposed to this parasite, of either 2×10^7 or 2×10^5, but not of 2×10^4, spleen cells from syngenic rats which had recovered from a primary *P. berghei* infection. When the spleen cells from the latter animals were kept at 47 C for 45 min they remained alive, but no longer were able to transfer protection. The capacity to transfer adoptive immunity was not found in spleen cells from adult rats capable of age immunity. On the other hand, this capacity was found in spleen cells from rats that had suffered a very transient parasitemia (< 1% peak parasitemia).

Index Key Words: *Plasmodium berghei*; adoptive immunity in rats; spleen cell transfer; cell-mediated immunity.

THE role of cell-mediated immunity (CMI) in the protection of rodents against attack by *Plasmodium berghei* has been the subject of recent investigations. Transfer of lymphoid cells in syngenic rats has been one of the tools used in this type of research (3, 10, 12, 13, 17). Some of the parameters connected with cell-transfer experiments in malaria have been elucidated. Nevertheless, many questions remain to be answered.

When the lymphoid cells used in transfer experiments are obtained from spleens of rats that have recovered from a primary attack by *P. berghei* [immune spleen cells (ISC)], a few viable parasites remain in the ISC suspension (3). Phillips (10) treated donors with sulphadiazine to eliminate the parasites from the spleen cell suspension. An alternate method of eliminating these parasites was sought in our laboratory. Spira & Alger (personal communications) found that parasitized blood heated at 47 C for 45 min lost all its infectivity. Therefore, the effect of this treatment on ISC suspensions was investigated.

Age immunity to *P. berghei* has been observed in adult rats (14, 19) and in adult mice (1). Spira et al. (16) demonstrated

* This investigation was supported by the Agency for International Development, U. S. Department of State, Contract No. AID/csd-1432.

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that treatment of adult Lewis rats with antithymocyte serum completely abolished age immunity in these animals. A similar effect was observed by Spira & Zuckerman (15) and by Zuckerman & Yoeli (20) in splenectomized rats. Because of the apparent involvement of the spleen in age immunity, the effect of transfer of spleen cells from adult rats (never exposed to the parasite) into young susceptible animals was investigated.

Serum from rats that have received a primary and several challenging inoculations of *P. berghei*-infected blood is known to afford some protection to susceptible animals (6, 7). The protection transferred by this hyperimmune serum was elevated with an increase in the number of challenges received by the donor.

In cell transfer experiments, the parasitemia of donors of ISC might reflect a similar relationship. The protective effect of spleen cells from donors with a very low parasitemia is described in this report.

MATERIALS AND METHODS

Parasites and Rats

The NK65D strain of *P. berghei* was used in all experiments. This strain is kept in our laboratory by blood passages in mice or rats or in Alsever's solution plus 10% (v/v) glycerol in liquid N_2 (−193 C). All the animals used were female syngenic Lewis rats (from Simonsen Laboratories, Gilroy, California).

The method used for preparation of spleen cell suspensions has been reported previously (3). All injections of spleen cell and parasitized blood preparations were intraperitoneal.

Viability Test and Cell Counts

The tryphan blue exclusion test was used to determine the viability of the spleen cells. Only viable cells were included in the counts given in this report. In all preparations the viability was 95% or better. Cells were counted in a hemocytometer. To eliminate red cells (RBC) from the spleen cell suspensions, 2% (v/v) acetic acid was used as the lysing agent.

Challenge

All experimental rats were challenged 5 days after the injection of spleen cells with 10^6 RBC infected with *P. berghei*.

Parasite Count

Blood films were taken every other day by tail bleeding and fixed in absolute methanol after being air dried. They were stained with Giemsa's. The percent parasitemia was calculated by counting the number of infected cells in ~500 RBC.

Statistical Methods

The completely randomized design (18) and the Bayes least significant difference test (5) were used. The comparisons were made by calculating the average percent parasitemia (APP), defined as the summation of all the percent parasitemias for each day the blood films from the animal contained *P. berghei*-infected RBC, divided by the number of days the control rats had a patent parasitemia. If the rat died, the summation of the percent parasitemias was divided by the number of days it survived with a positive parasite count.

The peak percent parasitemia (PPP) is taken as the highest percent of infected RBC found in a particular animal during the course of infection. PPP was ~35% and occurred between post-inoculation days 12 and 14. The patent period (PP) of *P. berghei* in the 28-35-day-old Lewis rats used in the experiments was ~16-18 days.

Protected Rats

Throughout the experiments an animal was considered protected if after inoculation with 10^6 *P. berghei*-infected RBC, it met at least 2 of the following requirements: A) the PPP was 40% or lower than that of control groups (i.e. < 14%). B) The PP was ½ or less than proper controls (i.e. < 8-9 days). C) The APP was ⅓ or lower than controls (i.e. <5-6%).

Donor Rats

Experiment I.—The donors of ISC for both parts of this experiment were 38 days old when injected with 10^6 *P. berghei*-infected RBC. They recovered from the disease after a 30-40 PPP and an 18 day PP. Spleens were obtained from these rats 15 days after the last positive blood film was taken (usually at 71 days of age). Rats never exposed to *P. berghei* served as donors of normal spleen cells (NSC). They were of the same age as the ISC donors.

Experiment II.—Group **1.** 276-day-old rats that had never been exposed to *P. berghei*. Group **2.** 383-day-old rats that had never been exposed to *P. berghei*. Group **3.** 273-day-old rats that had recovered from a primary attach of *P. berghei* when 59 days old.

Experiment III.—Group **1.** 84 to 91-day-old rats that had been injected with 10^6 *P. berghei*-infected RBC when 56-63 days old. At the time these animals were infected they showed age immunity. The PPP was less than 1%, the PP less than 8 days, and the APP under 0.5%. Group **2.** 56 to 63-day-old rats that had been treated with 10^8 ISC when 28-35 days old and challenged 4 days later with 10^6 *P. berghei*-infected RBC. These animals had the PPP, PP, and APP similar to those of Group 1. Group **3.** Donor rats in this group included animals of the same age as those in Group 2. They did not, however, receive ISC. The PPP in these rats was > 30%. The donors in this group are equivalent to those used in Experiment I. Group **4.** Rats of the same age as the animals in Groups 2 and 3 never exposed to *P. berghei*.

RESULTS

Experiment I

Part 1.—The recipient rats, 31 days old, received varying numbers of ISC or of NSC (Table 1). The different numbers of cells all were administered in 1 ml volumes of inoculum. Three of the 8 groups received ISC previously placed in 1 ml aliquots in 10 × 75 mm test tubes and heated in a 47 C water bath with gentle agitation for 45 min. The protective capacity of ISC was apparent in recipient groups D and E which were injected with the 2 highest doses (Table 1). None of the 6 groups of animals that received the smallest amounts, 2×16^6, of ISC could be considered as protected. The PPP was approximately the same (6%) in all the protected animals despite the ISC number they received.

Regardless of the number of ISC injected, no protection was observed in rats receiving heated cells (Table 1). Nevertheless, the heat proved to be ineffective in killing all the parasites. This was proved by injecting 1 ml aliquots of the heated ISC suspension into highly susceptible mice. Half of the mice injected died of *P. berghei* infection; the other half had no parasites in the blood. A control group of mice was injected with similar amounts of nonheated ISC suspension. All the animals in this group died of the infection. The heat treatment, on the other hand, did not kill the ISC or the NSC. No noticeable decrease in live, nucleated cells in the spleen cell suspensions could be demonstrated by tryphan blue viability tests before and after this treatment.

TABLE 1. *Effects of transferring different numbers of heated immune (ISC) and normal (NSC) spleen cells on the course of infection with* Plasmodium berghei *in Lewis rats. The challenge (10^4 infected RBC) was administered 5 days after spleen cell transfer.*

Group	Spleen Cells No. injected	Spleen Cells Nature of	Spleen Cells Heat treatment*	Rats No. protected/ No. in group	Mean ave. % parasitemia†	Rats No. dead/No. unprotected
A	2	Immune	+	0/6	16.47	1/6
B	.2	Immune	+	0/6	17.02	2/6
C	.02	Immune	+	0/6	11.45	0/6
D	2	Immune	−	4/6	.63	1/2
E	.2	Immune	−	5/5	4.75	0/0
F	.02	Immune	−	0/6	15.91	2/6
G	2	Normal	−	0/5	20.01	1/5
H	—	−	−	0/5	19.25	1/5

* 47 C for 45 min (Tables 1, 2); † Bayes L. S. D. ($P > 0.1$) = 11.13.

TABLE 2. *Effects of transferring immune (ISC) and normal (NSC) spleen cells heated in siliconized test tubes on the course of infection with* Plasmodium berghei *in Lewis rats. All animals received 2 × 10^4 spleen cells and were challenged 5 days later with 10^4* P. berghei*-infected RBC.*

Group	Spleen Cells Nature of	Spleen Cells Heat treatment*	Test tubes	No. protected/ No. in group	Mean ave. % parasitemia†	Rats No. dead/No. unprotected
A	Immune	+	S‡	0/7	20.17	7/7
B	Immune	+	NS§	0/7	27.16	7/7
C	Immune	−	S	5/7	.28	2/2
D	Immune	−	NS	4/7	.67	3/3
E	Normal	+	S	0/7	22.31	7/7
F	Normal	+	NS	0/7	23.86	6/7
G	Normal	−	S	0/7	29.22	6/7
H	Normal	−	NS	0/7	26.53	7/7

† Bayes L. S. D. ($P > 0.1$) = 10.35; ‡ siliconized; § nonsiliconized.

Part 2.—Because the results obtained with heated and unheated cells in the above experiment could be attributed to the selective depletion of an adherent population of lymphocytes (8) another experiment was performed.

The design was similar to that used in the first part (Table 1), except that only a single dose, 2 × 10^8, of spleen cells was injected (Table 2) and 28-day-old rats were used as recipients. Half of the groups received spleen cells that had been heated in 10 × 75 mm test tubes siliconized with a diluted solution of Siliclad (Clay Adams, Division of Becton, Dickinson & Co., Parasipparity, N.J. 07054). The silicone coating inhibited adhesion of the cells to the glass.

Rats receiving cells heated in siliconized or nonsiliconized test tubes were completely unprotected. This suggests that the suppression of the adoptive immunity to *P. berghei* is dependent on a population of cells that are sensitive to 47 C heat.

Experiment II

When recipient animals (34 days old) were treated with ISC they showed a mean APP of 2.18 (Table 3). This figure was significantly ($P > 0.01$) lower than those recorded from Group A and B rats treated with spleen cells from donors known to have age immunity. The results from the latter 2 groups were not significantly different from those obtained in normal controls (Group D).

Experiment III

It is evident from the results of this experiment that in spite of the parasitemia observed in the donor of the ISC (< 1% or > 30%), these cells protect the recipient (Groups A, B, and C) against a challenge with *P. berghei*-infected RBC (Table 4). The recipients in this experiment were 35 days old.

The spleens obtained from donor Group 3 (Table 4) had signs of splenomegaly and heavy pigmentation known to occur in subjects suffering from malaria (4, 11). On the other hand, spleens isolated from donor Group 1 were enlarged only very slightly (not quantitated). Finaly, the spleens collected from donor Group 2 resembled in size and color the normal spleens from donor Group 4.

When the spleens were removed from Group 2 donors, a black mass was observed in the peritoneal cavity of these rats. Microscopic examination (at 1000 × magnification) of this mass revealed no cells. The mass consisted of malaria pigments, probably from the ISC injected into the animals (see Materials and Methods, Experiment III). In all the hosts it was adjacent to the hillus of the spleen, this site being diagonally opposite that of the ISC inoculation.

Positive blood films were obtained from Group A and C recipient rats shortly before the challenging parasites were inoculated. This indicates that parasites were present in the ISC suspension obtained from donor Groups 1 and 3. In contrast, no parasites were seen in blood films from any Group B recipient animals.

The course of infection in the Group A, B, and C rats was similar. All these animals had a very low PPP (4%) and APP (0.17-0.61%) and a short PP (4-8 days).

DISCUSSION

Experiment I

Dose effect.—The results obtained in this experiment are in agreement with those reported by Roberts (12) and substantiate his idea that before adoptive immunity is apparent in the *P. berghei*-rat system a certain level of parasitemia in the recipients (i.e. antigenic stimulus) has to be reached.

Heat effect.—The results summarized in Table 2 suggest that the ability of ISC to transfer immunity is affected by heat (47 C, 45 min). Immunologic studies on lymphoid cells treated with mild heat were carried out by Amiel & Mathe (2) and Mathe et al. (9). These investigators found that a 2-hr incubation at 37 C reduced the capacity of mouse spleen cells to engage in graft *vs* host reactions, although the number of viable cells remained the same. The procedure did not affect the ability of these cells to restore the hematopoietic capacity of lethally irradiated mice (9). Although these results and those reported herein are not comparable (differences in temperature, length of incubation, medium used to incubate the cells, and recipient used), the basic idea that heat affects the immunologic capacity of spleen cells is worth considering.

TABLE 3. *Effects of transferring immune (ISC) and normal (NSC) spleen cells obtained from donors of different ages on the course of infection with* Plasmodium berghei *in Lewis rats. The challenge inoculum (10^6 infected RBC) was given 5 days after transfer of 10^8 spleen cells.*

Recipient Group	Nature of Spleen Cells	Donor rats Group	Donor rats Age (days)	No. protected/ No. in group	Mean ave. % parasitemia†
A	Normal	1	(276 days)	0/8	10.36
B	Normal	2	(383 days)	0/8	11.02
C	Immune	3	(273 days)	7/8	2.18
D	—	—		0/6	12.78

† Bayes L. S. D. ($P > 0.1$) = 3.66.

TABLE 4. *Effects of transferring immune (ISC) spleen cells from donors with different degrees of parasitemia on the course of infection with* Plasmodium berghei *in Lewis rats. The challenge inoculum (10^6 infected RBC) was administered 5 days after transfer.*

Recipient Group	Nature of Spleen Cells*	Donor rats Group	Donor rats Age (days)	Donor rats % peak Parasitemia	Recipient rats No. protected/ No. in group	Mean ave. % parasitemia†
A	Immune	1	(84-91 days)	< 1	8/8	.41
B	Immune	2	(56-63 days)	< 1	8/8	.47
C	Immune	3	(56-63 days)	>30	4/6	.41
D	Normal	4	(56-63 days)	—	0/8	17.80
E	—	—		—	0/6	16.90

† Bayes L. S. D. ($P > 0.1$) = 8.74.

Experiment II

It has been suggested that age immunity is related to the availability of different RBC types (14). In the case of *P. berghei* the young animal is more susceptible, apparently because it has a higher percentage of reticulocytes in its blood stream. An adult rat, on the other hand, has a very low percentage of reticulocytes. As is well known, reticulocytes are preferred by *P. berghei* for erythrocytic development (14). These facts might explain the failure to transfer any age immunity with the spleen cells from adult rats. Since the RBC distribution of the young recipient rats were not affected by the injection of the spleen cells, the availability of reticulocytes for parasite invasion was not altered.

The suppression of age immunity by antilymphocyte serum (16) indicates that the phenomenon of age immunity in rats is related to thymus-dependent lymphocytes. In the experiment reported here spleen cells (thymus-dependent cells are abundant in the spleen) from adult rats were incapable of transferring age immunity. These results would suggest that age immunity might reside in the thymus-dependent areas found in organs other than the spleen (i.e. lymph nodes).

Experiment III

Immune spleen cells from donors that suffered a peak parasitemia of < 1% transferred the same degree of adoptive immunity as those from donors with peak parasitemias of ~30% (Groups A, B, and C, Table 4). In this respect, adoptive transfer of immunity to *P. berghei* differs from the protection transferred by hyperimmune serum. Fabiani & Fulchiron (6) and Fabiani & Orfila (7) found an increase in the protective effects of hyperimmune serum with an increase in the numbers of challenges.

The results of the present experiments support the idea that CMI is related to the immune response observed in rats against *P. berghei*. They do not exclude, however, the possibility that humoral immunity is also a part of this response.

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