Effect of Vitamin A and Undernutrition on the Susceptibility of Rodents to a Malarial Parasite *Plasmodium Berghei*[1]

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ABSTRACT   The ability of vitamin A deficient rats to resist infection with *P. berghei* was investigated. When 10×10^6 erythrocytes bearing the parasite/100 g body weight were given to the vitamin A protein energy undernourished rats, parasitemia developed in these animals at a faster pace than the controls. A high number (60% to 95%) of red blood cells (RBC) carrying the parasite were noticeable within 6 to 7 days after infection, at which time most animals in this group died. The pair-fed controls (protein-energy undernourished but supplemented with vitamin A) fared perceptibly better with an equivalent load of infection. Control ad libitum fed littermates were able to restrict the infection and neither high parasitemia nor death was noted in this group. Oral supplements of retinyl acetate to vitamin A deficient rats enabled the animals to recover from infection. A subclinical dose of 500 parasitized RBC given at an early stage of the vitamin A deficiency precipitated the deficiency symptoms at a faster rate and led to the development of higher order of parasitemia in these rats beginning from the 10th day after infection as compared to pair fed controls. The yield of glass adhering cells obtainable from peritoneal exudates was low in deficient rats. In vitro experiments further suggest a decrease in the capacity of the glass adhering peritoneal exudate cells in vitamin A deficient mice to clear the infection. This capacity was improved by addition of non glass adhering cells from sensitized control mice.   J. Nutr. *106:* 784–791, 1976.

INDEXING KEY WORDS   parasitemia · vitamin A · energy deficiency · phagocytic cells

Vitamin A is attributed to endow the body with protective capacities. In deficiency states of this vitamin, bacterial (1, 2, 3) and viral infections (4) are more pronounced, and at times fatal. The protective effect of the vitamin is presumably exercised at more than one level. In its absence or relative deficiency, the epithelial linings are not in an optimal functional state. A decrease in the synthesis of mucopolysacchrides and mucous secretions has been reported (5), although other investigators failed to observe an effect of vitamin A deficiency on incorporation of [35S]sulphate into mucopolysacchrides (6). In vitamin A deficiency which in the field is invariably accompanied by protein energy undernutrition, the turn over and number of neutrophils and other types of cells required for handling the infective agent is diminished (7). Vitamin A deficiency can also impair the host immune responses. Indeed a significant decrease in

the ability of vitamin A, protein and energy deficient animals to produce antibodies against a variety of antigens has been observed (8). The cell mediated immune processes are also notably impaired.[3] In view of the fact that vitamin A deficiency is widely prevalent among children in many parts of India where the population is exposed to a variety of parasitic diseases, it was considered relevant to assess the defense potential of vitamin A deficient animals against a malarial parasite. *Plasmodium berghei* is a parasite adapted to rats. When injected into a well nourished rat, it multiplies in the infected animal but regression sets in after a few days and the rat is able to overcome the infection (9, 10). The host-parasite interactions have been studied in experimentally produced vitamin A deficient rats.

MATERIALS AND METHODS

Production of experimental vitamin A deficiency. The experiments were conducted in A.I.I.M.S. bred male rats derived from Holtzman strain and in mice (Swiss strain). The rats were housed individually whereas mice were kept in groups in plastic cages.[4] The animals were maintained at room temperature ranging from 22° to 26° and having 14 hours light and 10 hours dark. At weaning (21–22 days of age), litters weighing 25 to 35 g were divided into three groups. Group 1 was fed the vitamin A free diet[5] ad libitum, group 2 was fed the same diet ad libitum but given oral supplements of retinyl acetate (50 μg/day) and group 3 rats were pair-fed controls that received an amount of diet equivalent to that consumed by group 1 animals, and were given oral supplements of retinyl acetate (50 μg/day). Retinyl acetate[6] was dissolved in petroleum ether added to groundnut oil and given orally where indicated with the help of a tuberculin syringe. The animals were weighed weekly. The growth pattern of the three groups of rats is given in figure 1. The initial consumption of diet of the rats was 4 to 6 g/day. It increased to 15 to 18 g in the group of control ad libitum fed animals but was a maximum of 10 to 12 g/day in vitamin A deficient animals. The animals were considered vitamin A-depleted when their body weights became
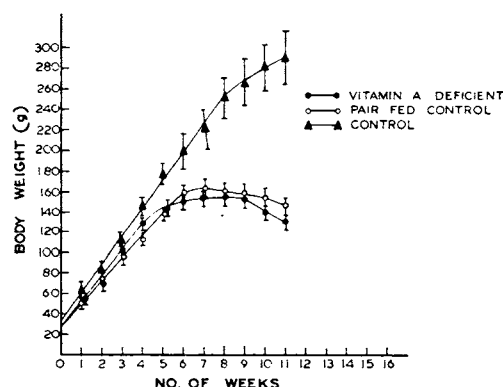

Fig. 1 The growth pattern of vitamin A deficient, ad libitum fed and pair-fed rats. Littermates were divided in three groups and fed the diet as described in methods. The points give a mean ± SEM. ($n = 5$).

stationary, or tended to fall and xerophthalmia was noticeable. Vitamin A deficiency was confirmed in all cases by the absence of autofluorescence in cryostat sections of liver and by the estimation of liver vitamin A content (11) at the time of killing or death, which was reduced in deficient animals to a limit undetectable (<1 μg in total organ extract).

Production of P. berghei infection. The strain of *P. berghei*[7] used for infecting the animals was maintained in Holtzman rats or in Swiss strain mice by weekly passage of parasitized erythrocytes (30×10^6) in rats or (10×10^6 erythrocytes) in mice. At the end of about 1 week, heparinized blood from the tail of animals in which parasitemia was about 80% was taken and used as inoculum for passaging.

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[3] Savithri Krishnan (1975) Effect of vitamin A deficiency and concomitant protein calorie undernutrition on defence mechanisms. Ph.D. Thesis, All India Institute of Medical Sciences, New Delhi, India.

[4] Maryland Plastic Inc.

[5] The U.S.P. XIV vitamin A test diet was used with slight modifications. The composition was as follows: Casein (refluxed with ethanol and extracted with ether), 18%; starch, 63%; sucrose, 10%; refined ground nut oil, 5%; salt mixture (17), 4%. The following vitamins were added per kg of diet: α-tocopherol, 100 mg; 2-methyl-1,4 naphthaquinone, 5 mg; ergocalciferol, 5 mg; thiamin, 5 mg; riboflavin, 5 mg; nicotinic acid, 50 mg; pyridoxine, 5 mg; pantothenic acid, 50 mg; biotin, 0.5 mg; folic acid, 0.5 mg; inositol, 100 mg; choline chloride, 1 g; ascorbic acid, 1 g.

[6] Obtained from Hoffmann La Roche, Basle (Courtesy of Dr. Otto Isler).

[7] Obtained from the National Institute of Communicable Diseases (NICD), New Delhi (Courtesy of Dr. P. N. Saigal, Dr. M. I. D. Sharma, Dr. M. M. Mondal and Dr. N. G. S. Raghavan).

Parasitized red blood cells (RBCs) obtained from animals in which 80% parasitemia had developed were used as the infecting inoculum. Ten million erythrocytes parasitized with P. berghei per 100 g body weight were injected intraperitoneally into the experimental animals.

Thin blood smears were prepared from the tail blood of individual rats daily throughout the course of infection. The slides were dried, fixed in methanol and stained with eosin and polychrome methylene blue according to the method of Jaswant Singh (12). Five hundred erythrocytes were counted and the percent parasitemia calculated from the proportion containing the parasites.

Sensitization of vitamin A deficient and control mice with irradiated P. berghei. The inoculum for sensitization of the animals was prepared as follows. The heparinized blood was collected from the tail of rats with about 80% parasitemia under sterile precautions. The parasites were released from the RBC by continuous freezing and thawing and were exposed for 30 minutes to UV radiations from a germicidal lamp.[8] The parasites released from 10 million infected RBCs were injected intraperitoneally into the experimental mice.

Culture and infection of peritoneal exudate glass adhering cells (GAC). Peritoneal exudate cells were collected asceptically by peritoneal lavage with Ham F-10 medium.[9] One million cells in 0.1 ml of the medium were layered on coverslips in Leighton tubes containing 1 ml of the medium with 20% fetal bovine serum. Incubation was done at 37° in the presence of 5% CO_2 and 95% air.

The glass adhering cells (which includes macrophages) were infected with parasites released from about 5×10^6 infected erythrocytes. The inoculum was prepared by continuous freezing and thawing of the heparinized blood for 30 minutes. After 1 hour of infection, the cultures were washed free of the unphagocytosed parasites and the clearance of P. berghei from the GAC (mostly macrophages) was studied by counting at various time intervals. For this purpose, the coverslips were taken out of the Leighton tubes and washed twice with phosphate buffered saline (pH 7.2), fixed in 3% glutaraldehyde and stained with eosin and polychrome methylene blue. One to two hundred cells were counted and the percentage of infected cells calculated. Where indicated, serum from sensitized mice or non glass adhering peritoneal exudate cells containing the lymphocytes were added to the cultures.

RESULTS

Response of vitamin A deficient and control rats to infection by P. berghei. Parasitized erythrocytes ($10 \times 10^6/100$ g body weight) were injected into vitamin A deficient, pair-fed and control group of rats. The time kinetics of parasitemia in one set of rats is shown in figure 2. Nine sets of experiments were performed. The following deductions can be made from these experiments.

Parasitemia increased at a faster rate in the vitamin A deficient rats as compared to the other two groups. High parasitemia was attained in the deficient rats in each of the nine sets of experiments, the values varied in this group (table 1) from 20% to 100% of erythrocytes in peripheral circulation bearing parasite. In the pair-fed group, the parasitemia ranged from 8% to 60% with a mean of 27 ± 6.2 (SEM). In the control ad libitum fed rats, 5% to 40% (mean 16 ± 4.4) of erythrocytes bearing
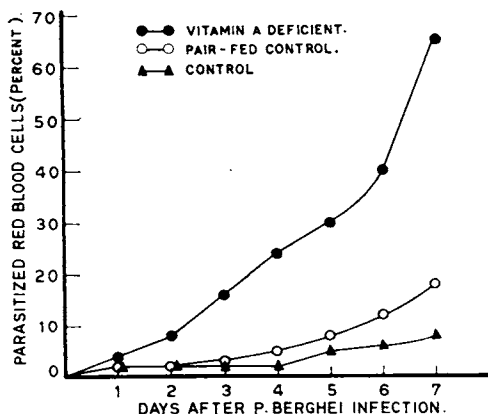

Fig. 2 Infectivity pattern of vitamin A deficient rats and controls after infection with *Plasmodium berghei.*

TABLE 1

*The percentage of erythrocytes bearing parasites[1]
in circulating blood of vitamin A deficient,
pair-fed and ad libitum fed rats*

Experiment no.	Vitamin A deficient	Pair fed	Control
	%	%	%
1	60	8	5
2	30	10	8
3	20	15	5
4	65	12	6
5	40	15	5
6	95	40	20
7	95	35	25
8	100	60	40
9	95	45	30
Mean ± SEM	67 ± 10.4^a	27 ± 6.2^a	16 ± 4.4^b

[1] Means not sharing a common superscript letter differ significantly, ($P < 0.01$), by Student's *t*-test.

parasites were noted. The vitamin A deficient group was significantly different than the control and the pair fed group. There was no significant difference in parasitemia between the control and the pair fed group.

Seven of the vitamin A deficient rats died on day 6 or day 7 of the infection although in two of the nine experiments, the rats died on the 4th or 5th day of infection. The blood smear taken on the day of the death of the deficient rats shows heavy parasitemia (fig. 3B). For comparison blood smears from the control and the pair-fed rats on the same day of infection are also included (figs. 3A & 3C respectively). None of the rats in the control group died during the 5 week observation period. The survival time of pair-fed animals with an equivalent load of infection was between 9 and 11 days.

Response of rats in early phases of vitamin A deprivation to low doses of parasitic infection. In the above experiments, the infection was introduced at a time when complete vitamin A deficiency had been attained. In clinical practice, one encounters partial deficiency states much more frequently than total deficiency states. It was thus considered appropriate to determine the ability of animals in partial states of vitamin A deficiency to resist a subclinical load of infection. Two weeks after the weaning period when the

diet of the rats was changed to be vitamin A deficient, a group of rats were infected with 500 RBCs parasitized with *P. berghei*. The progress of the infection in these rats was followed and compared with the pair-fed and control rats. Data given in figure 4 show that the rats fed the vitamin A deficient diet responded in a manner similar to the pair-fed controls up to about
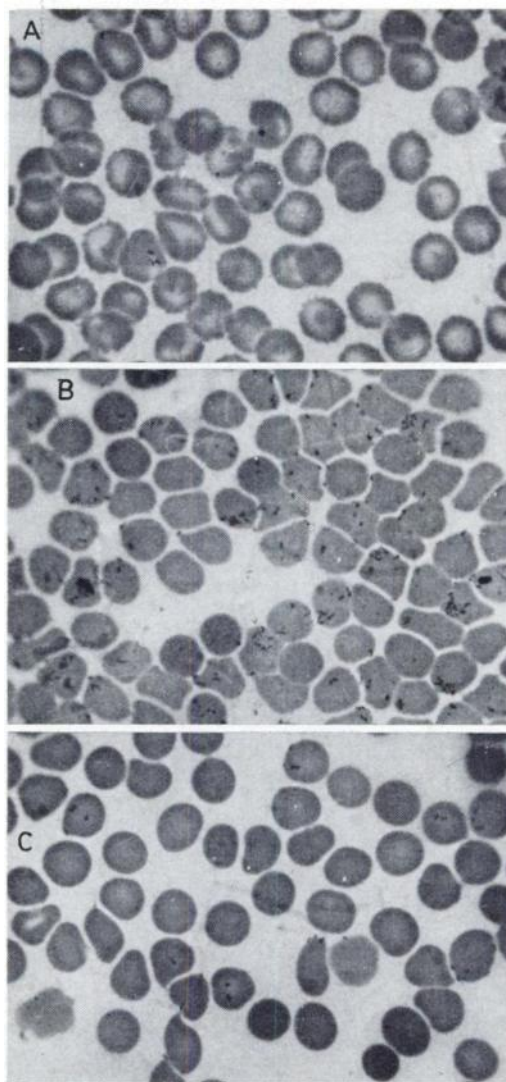

Fig. 3 Red blood cells from (A) control rat, (B) vitamin A deficient and (C) pair-fed control, 6 days after infection with *P. berghei*. (Eosin and Polychromemethylene blue, × 2,800). Many parasites are seen in the RBCs of the deficient rat.
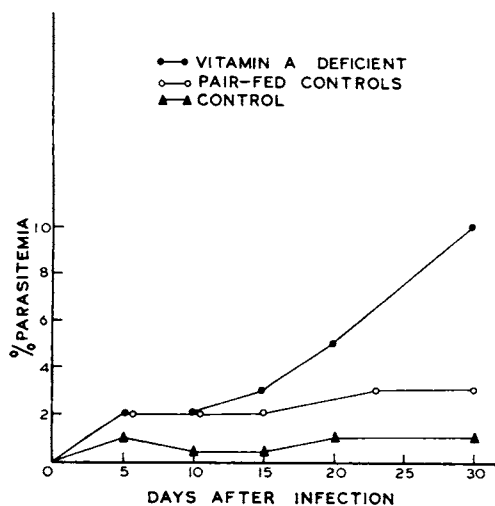
Fig. 4 The percentage of erythrocytes bearing parasites on various days following a subclinical infection with *P. berghei* in rats fed vitamin A deficient diet. ($n = 2$).

10 days after the infection. Thereafter, the parasitemia rose markedly in the rats fed the deficient diet. They died on day 30 after infection even though the percent parasitemia was approximately 10%. None of the rats in the pair-fed and control series died during the 5 week period ob-
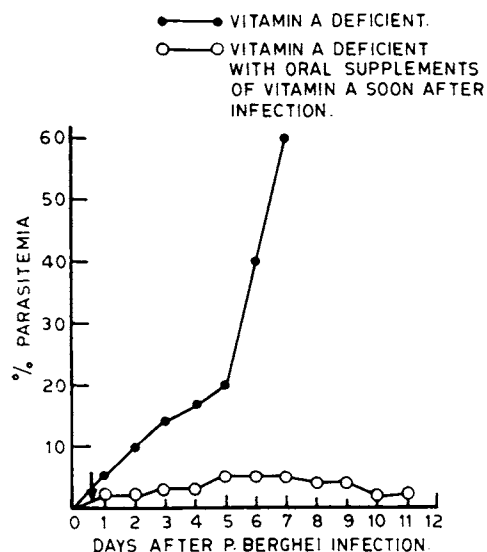

Fig. 5 Effect of vitamin A supplements (↓) on vitamin A deficient rats infected with *P. berghei.*

servation. The parasitemia developed in these groups was also of a mild order.

Recovery with vitamin A supplements. In order to confirm the important role of vitamin A in combating infection, vitamin A deficient rats were divided into two groups. Both groups (two rats in each) were infected with 10 million RBC containing *P. berghei.* From the day of infection, group 1 rats received oral supplements of vitamin A (50 µg retinyl acetate/rat/day). The progress of infection was studied in the two groups. The parasitemia on various days in these rats is shown in figure 5. Both rats which were given oral supplements of vitamin A recovered from the infection and survived for more than 5 weeks.

In vitro studies on the clearance of phagocytosed Plasmodium berghei by glass adhering peritoneal exudate cells. The total number of peritoneal exudate glass adhering cells (GAC) which could be recovered from vitamin A deficient mice were about 3×10^6 on an average in comparison with 20×10^6 in the control mice. The peritoneal macrophages from vitamin A deficient and ad libitum controls appeared similar in their capacity to spread on the glass as well as to phagocytose *P. berghei.* The cells from either of the two groups phagocytosed the parasite poorly. On an average about 20% of the cells phagocytozed the parasite. However in mice sensitized with irradiated parasites, the phagocytic index of the peritoneal exudate GAC 2 weeks after sensitization increased to about 60% in both vitamin A deficient and control mice.

The sensitized macrophages from the control mice bearing the parasites when incubated at 37° eliminated most of the phagocytized *P. berghei* in 72 hours (fig. 6A). On the other hand 70% of the macrophages from the vitamin A deficient mice had parasites in them at that time (fig. 6B).

To study the role of antibodies in the clearance of *P. berghei,* the infected GAC cultures were incubated in the presence or absence of serum from sensitized mice. The results given in figure 7 show that the serum from neither the deficient nor control mice exercised a significant effect on the clearance of the parasites from the glass adhering cells. When the cultures of
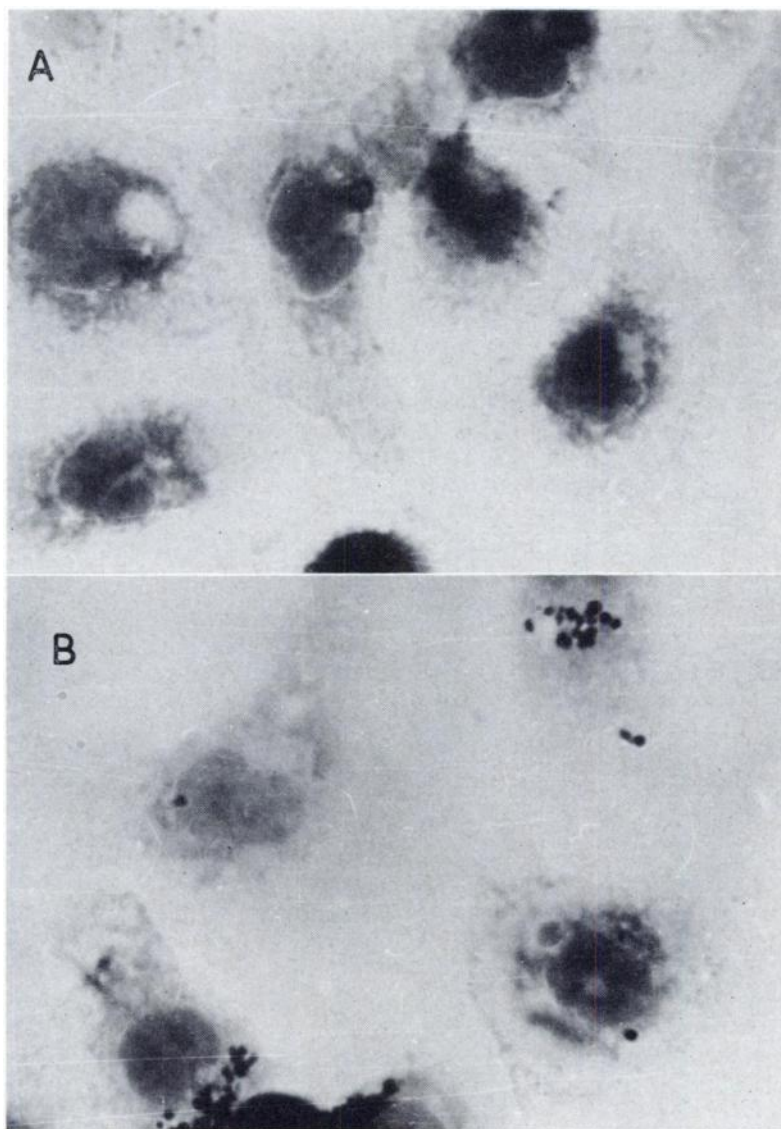
Fig. 6   Peritoneal exudate glass adhering cells from (A) control and (B) vitamin A deficient sensitized mice, after 72 hours of infection *in vitro* with *P. berghei*. (Eosin and polychrome methylene blue, × 3,500). No parasites are seen in the control macrophages, whereas parasites are abundant in the macrophages from deficient mouse.

glass adhering cells infected with *P. berghei* were incubated with nonadhering cells (lymphocytes) from sensitized vitamin A deficient mice, these cells did not have a major role in the clearance of the parasite from the GAC of either vitamin A deficient or control mice (figure 8). However, when the peritoneal exudate nonadhering cells from control sensitized mice were added to the GAC cultures from vitamin A deficient mice, there was a marked reduction in the number of infection bearing macrophages.

DISCUSSION

These investigations point to the beneficial role of both vitamin A and protein-energy nutrition in the capacity of the ani-

IN VITRO ELIMINATION OF P. BERGHEI
BY GLASS-ADHERING CELLS

- •———• D GAC
- o———o D GAC.& D SERUM.
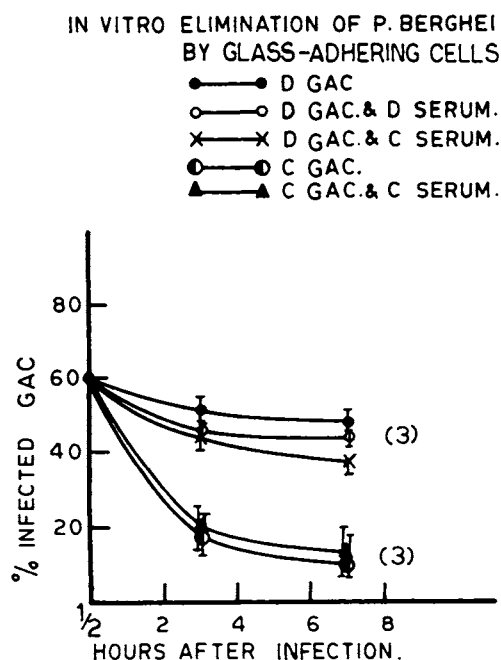- ×———× D GAC.& C SERUM.
- ◐———◐ C GAC.
- ▲———▲ C GAC.& C SERUM.

Fig. 7 The effect of serum from sensitized control (C) and vitamin A deficient (D) mice on the rate of elimination of *P. berghei* in macrophages derived from control (C) and vitamin A deficient (D) animals (Mean ± SEM values for three experiments).

mals to resist a parasitic infection. Animals with both vitamin A and a protein-energy deficiency demonstrated a total loss of resistance. The infective agent proliferated without the usual delay. Unrestricted growth of the parasite resulted in the death of the animal within a short period. In the protein-energy deficient animals which received oral supplements of vitamin A, there was a longer lag period suggesting a better initial resistance offered by these animals to the infective agent. The rate of growth of the parasite was also lower than was the case in vitamin deficient animals. The animals however lost the capacity to recover from infection and ultimately succumbed to it although at a later date than the deficient animals.

The important role of vitamin A in the defence potential of the animal is further indicated by the observations that oral supplements of the vitamin led to the recovery of animals which were deficient when infected.

The role of vitamin A in immunity may reside in its protective action at more than one level of host parasite interactions. The integrity of the epithelial linings and the normal state of mucous secretions are among the factors that may influence the invasion of a variety of infections. In addition, there is indication of a loss of the ability of phagocytic cells to clear the infection in the deficient animals. Similar data are available in malnourished children where bactericidal activity of the phagocytic cells was significantly decreased (13), and phagocytic activity was comparable to the controls in malnourished children and patients (13, 14). Other investigators have reported diminished phagocytic activity in protein deficient monkeys (15, 16). Experiments reported in this communication suggest that the disability of phagocytic cells to clear the infection in deficient animals is due to at least two factors. There is a lack of generation of phagocytic cells. The number of GAC obtainable from the peritoneal exudate of deficient animals was much lower than was the case with the normal animals. The deficient animals also showed a poor re-

IN VITRO ELIMINATION OF P. BERGHEI
BY GLASS-ADHERING CELLS

- •———• D GAC
- o———o D GAC.& D LYMPH.
- ×———× D GAC.& C LYMPH.
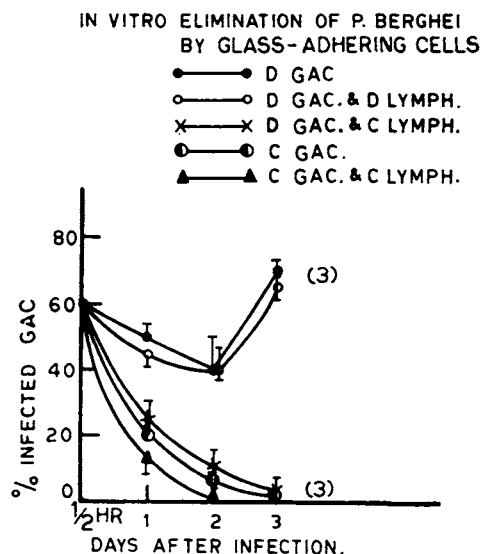- ◐———◐ C GAC.
- ▲———▲ C GAC.& C LYMPH.

Fig. 8 The effect of lymphocytes from sensitized control (C) and vitamin A deficient (D) mice on the rate of elimination of *P. berghei* in macrophages derived from control (C) and vitamin A deficient (D) mice.

sponse to immunization or sensitization with irradiated parasitic antigens. Though sensitization improved the capacity of the GAC from both deficient and control animals to phagocytose the parasites, the capacity to clear the phagocytosed parasite was poor in the deficient animals. While complete answers are not yet available to delineate the factors implicit in the clearing capacity of the GAC, a role of the non-adhering cells (NAC) is indicated by experiments reported in figure 8. Serum appears to have little role in clearance of the phagocytosed parasite.

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