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Immunoregulatory Consequences of Vitamin Deficiencies on Background Plaque-Forming Cells in Rats (39388)

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The detrimental effects of specific vitamin-deficiency states on the immune response have been known for some time (1). The pronounced impairment of the immune system has been well documented in both pyridoxine- and pantothenic acid-deficient rats (1). Defects in immune capacity in such deficient animals can be attributed neither to general inanition nor to impaired reticuloendothelial function in antigen distribution, but rather to decreased numbers of hemolytic antibody-producing cells as measured in plaque-forming cell assays (2) in rats responding to sheep erythrocytes (3, 4).

In an effort to extend these studies to animals injected with other antigens, and in order to approach questions regarding mechanisms of regulation of immune responses in general, and mechanisms of the immunological defects seen in vitamin-deficient rats in particular, studies were undertaken using targets in addition to sheep erythrocytes to evaluate the immune capacity of rats with specific vitamin deficiencies. In this report, we present results of the effects of riboflavin, pyridoxine, and pantothenic acid deficiencies upon background plaque-forming cells in nonimmunized rats.

Materials and Methods. Male, weanling albino rats of the Holtzman strain (Holtzman Co., Madison, Wisc.) were housed individually in wide-meshed, screen-bottom, suspended cages. They were weighed weekly and experiments were initiated 6 to 14 weeks after arrival at the laboratory.

Production of specific vitamin deficiences. All animals were fed a basal, semipurified diet *ad libitum* (4). In addition, each animal received a daily vitamin pill (4). Each of the pills fed to the control animals supplied adequate supplements of all of the B vitamins known to be required by the rat. For the riboflavin-, pyridoxine-, and pantothenic acid-deficient groups, only the respective vitamin was omitted from the control pill.

Assays for hemolytic plaque-forming cells. The following types of erythrocytes were used as targets in the direct hemolytic plaque-forming cell (PFC) assay (2): sheep red cells (SRC) from individual sheep were obtained in Alsever's solution from either Earl Cole, Green County, Pa.; Flow Laboratories, Rockville, Md., or Sacks' Farm, Evans City, Pa.; human O+ erythrocytes were obtained in ACD solution from the Central Blood Bank, Pittsburgh, Pa.; pooled rat red cells in Alsever's solution were obtained by cardiac puncture of donor normal rats of the same Holtzman strain as used in experiments.

Rats were sacrificed by a blow on the head and bled by cardiac puncture. Spleens and, sometimes thymuses, were removed aseptically, weighed, and dissociated by being pressed through a stainless steel wire mesh (2). Single cell suspensions were prepared (2); and aliquots of the resulting single cell suspensions containing 10^6 to 10^8 cells, depending on targets to be used, were subjected to hemolytic plaque assays (2) in 15 × 100-mm petri dishes. Preliminary experiments were run using more than one concentration of cells in order to determine appropriate cell concentrations. Base layers were not used in order to increase assay sensitivity; plaques were counted under 30× magnification using a dissecting microscope and indirect fluorescent light. A minimum of triplicate plates were counted for each type of red cell target used. Only those

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plaques fulfilling criteria for true PFC (2) were counted.

Results. The results of 10 experiments[3] involving rats with specific deficiencies of pantothenic acid, pyridoxine, or riboflavin are summarized in Fig. 1 and Tables I and II. The background PFC in nonimmunized rats directed against the selected erythrocyte targets were higher in the vitamin-deficient rats compared with controls in each of three specific vitamin deficiencies studied (Fig. 1). In contrast, we observed a markedly depressed response of PFC to immunization with SRC in riboflavin- and pantothenic acid-deficient rats.[4] The latter findings are in accord with previous observations in pyridoxine- (3) and pantothenic acid-deficiencies (4).

The increase in the numbers of background PFC per 10^8 spleen cells in the deficient groups was apparent despite the significant differences in whole body weight as well as in spleen weights in the deficient rats compared with control rats (Table I). Furthermore, not only is there an increase in background PFC per 10^8 spleen cells in the deficient groups, but there is an increase in the total numbers of such cells per spleen, as shown in Table II. This is true even through the spleens of deficient rats are strikingly smaller than control spleens (Table I). The ratio of spleen weight to body weight does not vary significantly between deficient groups and their respective controls. The number of cells per milligram of spleen also remains constant (1 million cells per mg) for each group. Thus, there is a definite overall increase in background PFC and not merely a relative increase of these cell populations compared with other spleen cells.

In experiments in which PFC in thymuses were also determined on the same erythrocyte targets as used in spleen PFC assays, the numbers of background PFC in thymuses of vitamin-deficient rats were also equal to or higher than the numbers of PFC in comparable control rats.

The increase in background PFC was found in PFC directed against all targets tested. The reactivity of PFC to SRC, human O+, and rat red cells (R) probably cannot be attributed to a shared antigen reactivity. The SRC used in these studies came from three different suppliers, and although SRC blood groups were not determined, only one blood type among seven blood groups in sheep appear to be related to H of man (5). Moreover, in experiments in which vitamin-deficient rats as well as control rats

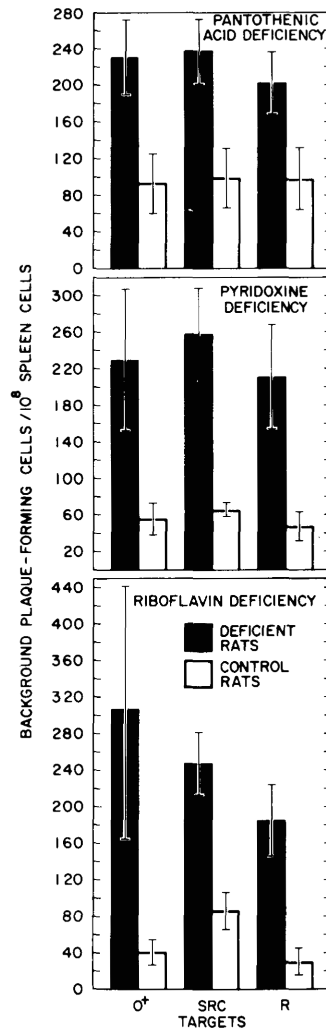

FIG. 1. Background plaque-forming cells (PFC) per 10^8 cells in spleens of six groups of rats against three different erythrocyte targets, human O+ red cells (O+) sheep red cells (SRC), rat cells (R). The numbers of rats in each group are those shown in Table I. PFC are shown as arithmetic means. Vertical bars represent ±1 SE.

[3] In each of 10 experiments, cell suspensions from experimental and control rats were run in parallel under identical laboratory conditions.

[4] Koros, A. M. C., Axelrod, A. E., Hamill, E. C., and South, D. J., unpublished data.

TABLE I. Body Weights and Spleen Weights of Vitamin Deficient and Control Rats.

Group	Number of rats per group	Rat weight (grams)[a]	Spleen weight (milligrams)[a]
Pantothenic acid-deficient	31	130.0 ± 9.0	425.9 ± 24.4
Controls	30	262.8 ± 12.9	836.3 ± 41.1
Riboflavin-deficient	17	73.0 ± 3.7	225.4 ± 12.0
Controls	12	214.8 ± 20.7	786.0 ± 58.6
Pyridoxine-deficient	21	102.1 ± 8.0	357.9 ± 17.7
Controls	25	237.3 ± 8.9	868.7 ± 49.6

[a] Arithmetic mean ± 1 SE.

TABLE II. Plaque-Forming Cells per Total Spleen in Vitamin-Deficient and Control Rats.[a]

	PFC per total cells per spleen[b]		
	Erythrocyte targets		
Group	O+	SRC	R
Pantothenic acid-deficient	908.8 ± 199.2	858.5 ± 149.3	818.2 ± 168.5
Controls	489.6 ± 220.3	516.8 ± 200.3	488.2 ± 185.2
Riboflavin-deficient	614.4 ± 219.6	608.9 ± 138.3	340.1 ± 52.5
Controls	277.1 ± 124.9	562.6 ± 132.9	172.8 ± 64.2
Pyridoxine-deficient	788.7 ± 412.5	757.4 ± 219.7	577.6 ± 154.2
Controls	301.5 ± 84.5	408.2 ± 66.9	245.0 ± 57.3

[a] The number of rats per group is the same as shown in Table I.
[b] Values based on actual cell counts (10) expressed as arithmetic mean ± 1 SE.

were inoculated with a single large dose of SRC, the control immunized rats responded dramatically to SRC when spleens were assayed on SRC targets, but exhibited no increase over background PFC when assayed on human O+ or R erythrocytes.[4] The numbers of postimmunization anti-SRC PFC in these immunized control animals were comparable to those found earlier (3, 4).

Discussion. Although the significance of an increased number of background PFC in certain vitamin-deficient states is not known presently, it is tempting to speculate that background PFC may reflect a unique regulatory parameter in both normal and immunologically impaired animals for the following reasons: (i) Suppressive 7S specific anti-SRC antibody, which markedly inhibits the appearance of postimmunization PFC directed against SRC, has no effect on background PFC directed against SRC (6). (ii) Background PFC directed toward different haptens appear at predictable times in a predictable sequence in ontogeny for particular strains of inbred mice (7). (iii) Background PFC exist in germ-free mice in numbers comparable to those found in conventional mice (8) and are probably not merely an indicator of prior exposure to cross-reacting bacterial antigens. (iv) The morphology of background PFC also differs from postimmunization PFC. Although the majority of direct (19 S) PFC directed against SRC are either blast cells, large lymphocytes, or plasma cells, whose numbers vary depending on the time after antigen injection, the background PFC are small lymphocytes (9–11). (v) Only a small percentage (<5%) of background PFC have been shown to be involved in DNA synthesis, compared with 55% of Day 4 postimmunization PFC which are actively synthesizing DNA (10). The background PFC thus appear to have different rates of proliferation than postimmunization PFC.

The present study cannot attempt to define the function of background PFC in absolute terms, but does present a comparative survey of their existence in certain vitamin-deficient states in outbred rats. Categorization of background PFC as members of either B or T cell populations as described for mouse models (12) would require the use of inbred rats.

Increases in background PFC, especially autoimmune PFC, have been reported by others in NZB mice precedeing the onset of autoimmune disease (13, 14). Increases in

background PFC have also been reported in aged mice (15), and further elucidation of the role of background PFC might offer a clue to the postulated link between autoimmune diseases and aging (15).

In light of recent findings of the functional heterogeneity of lymphoid cell populations, especially T cells (16), it is possible that in the specific vitamin-deficient rat populations studied in this report, there is an increase in background PFC directed against the specificities tested, whereas there is a decrease in the precursor population(s) of postimmunization PFC directed against the same specificities. Such a situation could be due to the loss of a population of cells which may function normally to suppress background PFC as well as to cooperate with precursors of postimmunization PFC. Although the possibility cannot be excluded that the background PFC are direct precursors of postimmunization PFC, it is suggested from the current study as well as others (3, 4) that there may be a lack of a helper population necessary for the increase in number of PFC following antigenic stimulation in vitamin-deficient animals even though the background PFC exist in comparable or even greater numbers in vitamin-deficient rats than in controls. Whether this results from an actual increase in the rate of proliferation of background PFC in vitamin-deficient animals or a selected survival advantage of such cells only in vitamin-deficient animals is not known. Indeed the mechanisms whereby the vitamin deficiencies in this study impair the immune response are not known. Pyridoxine deficiency is the only one of the three studied in this report that has been shown to affect DNA synthesis (17).

It is also possible that background PFC may be directed against different, cross-reacting specificities rather than the same target erythrocyte specificities to which postimmunization PFC are directed. Nevertheless, the fact that all background PFC are markedly elevated in the three categories of vitamin-deficient animals studied compared with control animals suggests that the numbers of background PFC are a parameter of a regulatory defect in all of the deficient animals.

The finding of PFC against rat red cells may also be the result of a loss of a population of suppressive cells which are postulated to normally function to keep self-reactive cells in check (18, 19).

It is hoped that further studies into the nature of background PFC and their relation to postimmunization PFC may lead to a greater understanding of normal regulatory mechanisms involved in the immune response.

Summary. Background hemolytic plaque-forming cells (PFC) directed against three erythrocyte targets were measured in rats with three different, specific vitamin deficiencies: riboflavin, pyridoxine, and pantothenic acid, as well as in control rats. The numbers of background PFC were found to be elevated in all three vitamin-deficiency states compared with controls, whereas earlier studies showed that postimmunization PFC were markedly depressed in those deficiencies tested. The significance of these findings is discussed in terms of a possible loss of suppressor cells.

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1. Axelrod, A. E., *in* "Modern Nutrition in Health and Disease" (R. S. Goodhart and M. E. Shils, eds.), 5th ed., Chapt. 15, pp. 493–505. Lea and Febiger, Philadelphia (1973).
2. Jerne, N. K., Henry, C., Nordin, A. A., Fuji, H., Koros, A. M. C., and Lefkovits, I., Transplant. Rev. **18,** 130 (1974).
3. Kumar, M., and Axelrod, A. E., J. Nutr. **96,** 53 (1968).
4. Lederer, W. H., Kumar, M., and Axelrod, A. E., J. Nutr. **105,** 17 (1975).
5. Rasmusen, B. A., Ann. N.Y. Acad. Sci. **97,** 306 (1962).
6. Henry, C., and Jerne, N. K., J. Exp. Med. **128,** 133 (1968).
7. Merchant, B., Inman, J. K., and Claflin, L., *in* "Genetic Control of Immune Responsiveness; Relationship to Disease Susceptibility" (H. O. McDevitt and M. Landy, eds.), pp. 195–203. Academic Press, New York (1972).

8. Nordin, A. A., Proc. Soc. Exp. Biol. Med. **129,** 57 (1968).
9. Henry, C., Kimura, J., and Wofsy, L., Proc. Nat. Acad. Sci. USA **69,** 34 (1972).
10. Koros, A. M. C., Mazur, J. M., and Mowery, M. J., J. Exp. Med. **128,** 235 (1968).
11. Siegel, B. V., Brooks, R. E., and Morton, J. I., Blood **35,** 386 (1970).
12. Edelman, G. M., *in* "Cellular Selection and Regulation in the Immune Response" (G. M. Edelman, ed.), pp. 1-38. Raven Press, New York (1974).
13. Diener, E., Int. Archs. Allergy **30,** 120 (1966).
14. Wilson, J. D., Warner, N., and Holmes, M. C., Nature New Biology **233,** 80 (1971).
15. Hildemann, W. H., and Walford, R. L., Proc. Soc. Exp. Biol. Med. **123,** 417 (1966).
16. Gershon, R. K., *in* "Contemporary Topics in Immunobiology" (M. D. Cooper and N. L. Warner, eds.), Vol. 3, pp. 1-40. Plenum Press, New York (1974).
17. Trakatellis, A. C., and Axelrod, A. E., Biochem. J. **95,** 344 (1965).
18. Mosier, D. E., J. Immunol. **112,** 305 (1974).
19. Staples, P. J., and Talal, N., J. Exp. Med. **129,** 123 (1969).

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