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Immunological Alterations in Rats Fed with Flour from the Palmyrah Palm (*Borassus flabellifer* L)[1] (41286)

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Abstract. The humoral and cell-mediated immune competence of rats fed a 25% palmyrah flour diet was examined. The humoral response was evaluated by determining hemagglutinating antibody titers and hemolytic plaque-forming cell counts in the spleen following immunization with sheep red blood cells. The cell-mediated immune response was evaluated by the uptake of tritiated thymidine by peripheral blood and splenic lymphocytes following mitogenic stimulation. The immune competence of animals fed the flour for 2 weeks was not significantly altered. After 7 weeks, however, a significant and consistent depression of the humoral as well as the cell-mediated immune response was observed. Peripheral blood lymphocytes from rats which had been fed a 100% flour diet for 1 week also failed to respond to PHA stimulation. It is possible that these immunological alterations are etiologically related to the malignant lymphomas which develop in rats after prolonged feeding with palmyrah flour. Palmyrah flour appears to be the only human staple food which has been demonstrated to produce significant alterations in the immune competence of experimental rats.

Flour prepared from the young shoot of the palmyrah palm (*Borassus flabellifer* L.) is a staple food in some Asian and African countries. This flour has been reported to produce hepatotoxic lesions in short-term (1) and long-term (2) feeding experiments in rats. Moreover, we noted that malignant lymphomas developed in rats following prolonged feeding on a flour diet (3). The tumors occurred predominantly in the abdominal cavity, and one rat also had a thymic lymphoma. It was possible that these tumors resulted from immunosuppression induced by a toxic constituent(s) in the flour, since one of the mechanisms leading to a higher incidence of tumors (especially malignant lymphomas) in man and animals is the impairment of the immune system (4–6). A neurotoxic factor from this flour has recently been partially purified and its effects described (7).

The purpose of this work was to evaluate the humoral and cell-mediated immune competence of rats fed amounts of palmyrah flour capable of inducing lymphomas in these animals. The results obtained are consistent with our prediction that the flour does have immunosuppressive activity.

Materials and Methods. *Flour.* Palmyrah palm flour was prepared by grinding boiled and sundried fresh young shoots which had been purchased from the market in the form in which they are normally consumed in Sri Lanka. The fresh shoots were visually free from fungal contamination, and no aflatoxin could be detected in them. After preparation, the flour was stored at 4° to prevent microbial spoilage. It was mixed with powdered rat pellets (F. E. Zuellig, Bangkok, Thailand) at a ratio of 1:3 by weight as previously reported in toxicological experiments (1–3).

Animals and feeding. Randomly bred young adult male albino rats weighing 150–200 g were obtained from the Animal Center, Faculty of Science, Mahidol University. These animals were kept in indi-

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vidual stainless steel cages at approximately 25° with light provided 12 hr/day. All animals were given normal rat pellets (powdered) and tap water *al libitum* for at least 2 weeks prior to experimentation.

Animals in the experimental groups were each given 20 g of palmyrah flour mixture per day and they consumed almost all the food provided. Any left over was discarded before new food was added. Age- and sex-matched animals receiving a corresponding amount of palymyrah-free pellet powder served as controls.

Immunization with sheep red blood cells. Sheep red blood cells (SRBC) were washed thrice with cold phosphate-buffered saline, pH 7.2, and then suspended in saline at a final concentration of 8×10^8 cells/ml. A total of 4×10^8 cells in 0.5 ml was injected intravenously into the tail vein while each animal was under ether anesthesia. Animals were bled through the abdominal aorta 4 days after the SRBC injection. The serum, separated from the blood at room temperature, was kept frozen at −20° pending analysis for antibody. The spleen was removed immediately after death for analysis of antibody-producing cells.

Determination of serum hemagglutinating activity. Total and 2-mercaptoethanol (ME)-resistant hemagglutinating antibody (HA) to SRBC was titrated by a microhemagglutination technique (8). Results were recorded after reaction mixtures were allowed to stand overnight at 4°. Titers were expressed as geometric means with 95% confidence limits. For statistical analyses, sera with negative results at a final dilution of 1:4 (the lowest dilution tested) were arbitrarily given a value of 1:2.

Quantitation of antibody-producing cells. A modification of the Jerne plaque technique for determining the number of antibody-producing cells was used essentially as described by Cunningham and Szenberg (9). Both the plaque-forming cells (PFC) per 10^6 mononuclear cells and the total number of PFC per spleen were determined for each animal. The results were expressed as arithmetic means.

Lymphocyte proliferative response (LPR) to phytohemagglutinin (PHA). Transformation of peripheral blood and splenic lymphocytes by phytohemagglutinin-P (Difco Laboratories, Detroit, Mich.) was analyzed by a microassay method (10). Triplicate tests were employed for each of the control and mitogen concentrations (33, 100, and 300 μg/ml) used. Lymphocyte suspensions were prepared from peripheral blood and spleen homogenates by Ficoll–Hypaque density gradients.

Each well of the flat-bottom microtiter plates used contained 2×10^5 viable mononuclear cells in RPMI 1640 culture medium supplemented with 5% pooled normal rat serum, 2 m*M* glutamine and 5×10^{-5} *M* mercaptoethanol. The cells were cultured either with or without (unstimulated controls) the mitogen and incubated at 37° for 24 hr in an atmosphere of 5% CO_2 before 0.5 μCi [^{3}H]thymidine was added. The cells were harvested 18–24 hr later with an automatic cell harvester (MASH III, Microbiological Associates, Bethesda, Md.) and the quantity of [^{3}H]thymidine incorporated, as determined in a liquid scintillation spectrometer, was used to calculate the stimulation indices (SI) for each of the mitogen concentrations tested. The results were presented to correspond with the SI from 100 μg/ml of PHA-P since this gave the maximum stimulation in normal controls.

In addition to the thymidine incorporation method, blast transformation of peripheral blood lymphocytes was also studied. The method used was the same as that described by Moorhead *et al.* (11).

Results. *Hemagglutinating antibody titers.* The results presented in Table I show that feeding the rats on a palmyrah flour diet for 2 weeks did not depress their antibody production. On the other hand, after 7 weeks of feeding, a significant depression ($P < 0.05$) of humoral responses was noted when compared with controls.

Quantitation of antibody-producing cells. Similar to the results with HA titers, there was no reduction in PFC counts for animals fed palmyrah flour for 2 weeks. On the other hand, with rats fed for 7 weeks, there was a significant reduction ($P < 0.01$) of the PFC in spleens (Table II). Two of the rats

TABLE I. EFFECT OF PALMYRAH FLOUR DIET ON THE TITERS OF HEMAGGLUTINATING ANTIBODY 4 DAYS AFTER IMMUNIZATION WITH SRBC

Duration of feeding	Number of animals	Mean titers	
		Total	ME resistant
Control	21	1:31 (1:4–1:53)[a]	Less than 1:4
2 Weeks	19	1:31 (1:12–1:79)	Less than 1:4
7 Weeks	7	1:12 (1:3–1:40)[b]	Less than 1:4

[a] Geometric mean with 95% confidence limits.
[b] $P < 0.05$ when compared with control.

in this group had PFC counts that were reduced to less than 3% of the mean control value.

Lymphocyte transformation by PHA. Based on the uptake of [^{3}H]thymidine, the LPR of lymphocytes from rats fed palmyrah flour for 2 weeks were not depressed (Fig. 1). Indeed the responses of peripheral blood lymphocytes in this group seemed slightly enhanced in comparison with the control value, although the difference was not significant at the 5% level.

After 7 weeks, on the other hand, the responses of peripheral blood lymphocytes to various concentrations of PHA were markedly depressed (Fig. 1). The depression was noted throughout the range of PHA concentrations tested. The mean SI of the peripheral blood lymphocytes of palmyrah fed rats was 34% of the control mean. The blood lymphocytes from three of these rats were almost totally unresponsive to PHA stimulation, with stimulation indices of 10.5, 1.9, and 0.06% of the control value. Unlike the blood lymphocytes, the response of splenic lymphocytes from test animals was not suppressed according to this test. In fact, a slight enhancement was noted in some animals and this brought up the mean SI of the whole group to 129% of the control (Fig. 1). It should be indicated further that the suppressed responses to PHA stimulation of blood lymphocytes observed by the thymidine incorporation method was confirmed by the results showing impaired blast transformation of blood lymphocytes from rats fed pure palmyrah flour diet for 1 week.

Discussion. The evidence presented in this paper shows that animals given a palmyrah flour diet for 7 weeks had a significantly depressed antibody response to SRBC (Tables I and II) and lymphoproliferative response of blood leukocytes to mitogenic stimulation (Fig. 1). These observations are consistent with our prediction based on the development of malignant lymphomas in these animals (3) that palmyrah flour may have immunosuppressive activity for rats after prolonged consumption. Although the data presented are not sufficient to explain the mechanism(s) of immunosuppression by palmyrah flour, we recently reported that aqueous extracts of the palmyrah flour were clastogenic when tested on human peripheral blood lympho-

TABLE II. EFFECT OF PALMYRAH FLOUR DIET ON THE NUMBER OF ANTIBODY-PRODUCING CELLS IN THE SPLEEN 4 DAYS AFTER IMMUNIZATION WITH SRBC

Duration of feeding	Number of animals	Splenic plaque-forming cells	
		PFC/10^6 mononuclear cells	Total PFC/spleen
Control	13	1727 ± 342[a]	43015 ± 9768
7 Weeks	7	440 ± 221[b]	7607 ± 2631[b]

[a] Arithmetic mean ± SEM.
[b] $P < 0.01$ when compared with control.

cytes (12). Because most chemical clastogens are known to depress mitotic activity (13), the immunosuppressive activity of the flour reported in this paper could well be related to its clastogenic property. Such a relationship should be investigated. On the other hand, it is possible also that the immunosuppressive property could result from a preferential effect of the flour on suppressor cell activity as the latter is known to play an important role in the regulation of the immune response. Our results showing differential effects of the flour on splenic and peripheral blood lymphocytes (Fig. 1) are consistent with this possibility. Work is now in progress in our laboratories to isolate, purify, and identify the toxic component(s) of the flour and test its biological activities, including those on the immune system. This step should be undertaken in order to rule out the possibility that these effects are due to contaminating substances in the flour, although thus far we have failed to detect aflatoxins, nitrosamines, pyrrolizidine alkaloids, mercury, arsenic, and pesticides in several batches of the flour used in this study (unpublished data).

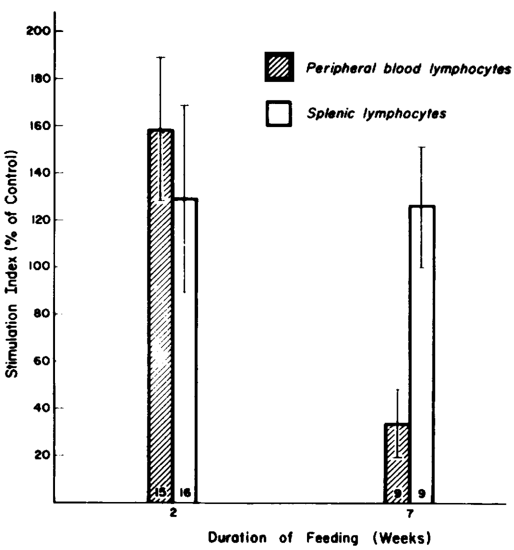

FIG. 1. Stimulation indices (SI) of peripheral blood and splenic lymphocytes from palmyrah-fed rats. Bars and lines represent mean ± SEM; the number at the base of each bar represents the number of specimens analyzed. SI were determined by the [^{3}H]thymidine incorporation method described under Materials and Methods.

The only datum available to us which is pertinent to the human situation and which may have some parallel with the development of lymphomas in palmyrah-fed rats is that the incidence of malignant lymphomas in the Northern Province of Sri Lanka is approximately three to four times higher than in the rest of the country (R. G. Panabokke, personal communication). It is in this region that palmyrah flour is extensively produced and consumed. Indeed in some families it replaces rice or wheat flour as the main caloric source and is consumed in amounts relatively greater than the amounts fed to the rats in our studies. The suggestion that this flour depresses immune competence may also be related to the relatively high incidence of leprosy in the Northern Province of Sri Lanka and in South India where this flour is also consumed. These observations merit further investigations into the possibility of alterations of immune competence in man by palmyrah flour.

Other plant products which have been reported to have immunosuppressive action in both experimental animals and humans include marihuana, steroidal lactones from *Withania somnifera* L. Dun (14) and vinca alkaloids. It is also noteworthy that plant lectins which are well documented to induce proliferative responses of lymphocytes may also be immunosuppressive, as has been shown for PHA (15) and concanavalin A (16). The immunosuppressive activity of navy bean lectin was demonstrated by Plouffe *et al.* (17) who noted a clustering of cases of Hodgkin's lymphomas in humans living in the vicinity of a navy bean processing mill. However, as far as we are aware, there are no reports of a natural food as extensively eaten by humans as the palmyrah flour, which produces immunosuppression and malignant lymphomas in experimental rats.

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