

Increased sensitivity to egg albumin in rats fed pristane

Phillip W. Albro, Richard O. Thomas, and Lawrence Fishbein
Research Triangle Park, N. C.

Rats fed a diet containing 1 per cent pristane for 5 weeks and then injected subcutaneously with egg albumin twice at weekly intervals developed a variety of pathologic conditions. The most characteristic effects were: seepage of blood into the pancreas, cecum, lungs, and/or bladder; fusion of the liver, spleen, pancreas, stomach, and diaphragm; and fatty degeneration of the lymph nodes. Rats surviving for a week after the second injection of albumin showed liver necrosis, fibrous intestinal tumors, and loss of splenic red pulp. Splenectomized rats, rats fed pristane but given only one injection of egg albumin, or rats given both injections of albumin but not fed pristane showed none of the abnormalities mentioned. The inclusion or omission of Freund's complete adjuvant with the albumin made no difference in the pathologic response. Rats fed pristane and given a single treatment with egg albumin showed less spleen-associated anti-egg albumin and fewer peritoneal mast cells than rats similarly treated with albumin but not fed pristane.

The detailed mechanism of action of adjuvants is not known, although there have long been a number of plausible hypotheses.¹⁻² Some antigens possess intrinsic adjuvanticity while others produce tolerance in the absence and immunity in the presence of extrinsic adjuvants. It has repeatedly been established that at least some adjuvants are effective when administered at times³ or by routes^{4, 5} different from that by which the immunogenic antigen is given. However, oral administration of substances effective as adjuvants when injected generally fails to result in adjuvanticity.⁶

Liquid paraffin (mineral oil), a necessary component of most adjuvants of the Freund's oil-in-water emulsion types, is absorbed to some extent by mammals on oral administration.^{7, 8} However, mineral oil without emulsifying agent apparently lacks adjuvanticity regardless of the route of administration.⁶ "Medicinal" mineral oil is a mixture of branched and cyclic, saturated aliphatic hydrocarbons. We have for some time used pristane and phytane as model compounds simulating most of the chemical and physical properties of light mineral oil while eliminating the difficulties of working with a multicomponent preparation. Pristane, a saturated 19-carbon isoprenoid hydrocarbon, is absorbed from the diet by rats to an extent of about 35 per cent of that fed.⁸ During part of our studies on the physiologic effects of orally ingested hydrocarbons, we have observed a striking effect of dietary pristane on rats immunized and challenged

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Reprint requests to: Phillip W. Albro, Ph.D., National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, N. C. 27709.

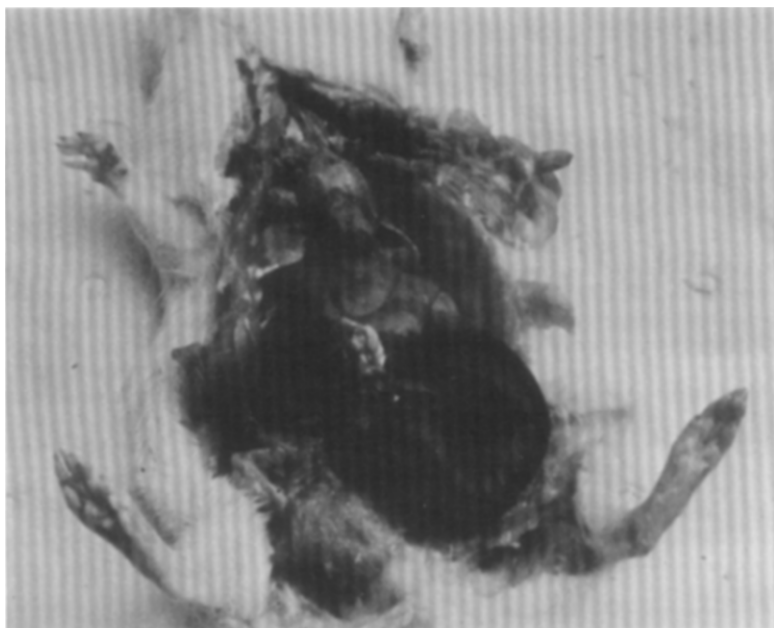


FIG. 1. Viscera of pristane-fed rat 48 hours after the second injection of egg albumin. Note cecum distended with clotted blood and very pale liver. From group 4A.

TABLE I. Summary of treatments

Group	Treatment	Number of rats	
		Pristane-fed (A)	Control (B)
1	Saline only	7	4
2	Albumin 2x, saline, splenectomized	2	2
3	Albumin 2x, Freund's adjuvant	15	8
4	Albumin 2x, saline	11	2
5	Albumin 1x,* Freund's adjuvant	3	3

*Group 5 did not receive the challenge with a second injection of egg albumin.

with egg albumin. Our exploratory experiments on the nature of this effect are presented here.

MATERIALS AND METHODS

Male CD strain rats (Charles River) were maintained on D&G Laboratory Diet (Price-Wilhoite Co.) throughout these experiments ("control diet"). "Treated diet" was prepared by letting pristane (2,6,10,14-tetramethyl-pentadecane, Eastman P7825) soak into the diet wafers. The concentration of pristane in the diet, one per cent by weight in all of the experiments reported here, was confirmed by extraction and gas chromatographic analysis⁸ of random one per cent samples of the treated diet.

Preliminary experiments revealed that at least 2 weeks were required for the levels of pristane in various rat tissues to stabilize after they had been assigned to the treated diet group. Unless otherwise specified, the rats used in these experiments were maintained on either control or treated diets for 5 weeks prior to inoculations beginning the immunologic study. The rats weighed 130 to 150 Gm. when first assigned to the two diet groups and 360 to 400 Gm. after maintenance on those diets for a period of 5 weeks.

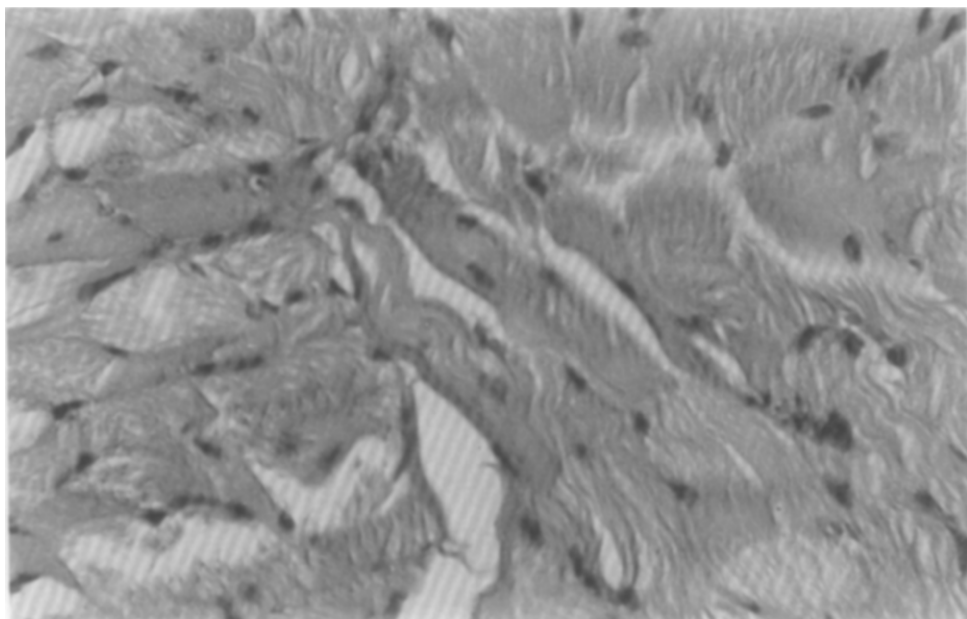


FIG. 2. Hematoxylin and eosin-stained section of intestinal tumor. Such fibrous tumors were conspicuous in several of the group 3A and 4A rats one week after the second injection of egg albumin. (x450.)

TABLE II. Pristane content of rat tissue*

Tissue	Pristane ($\mu\text{g}/\text{Gm. wet weight}$)
Liver	136
Spleen	100
Lymph node†	240
Pericardial fat	160
Epididymal fat	363
Subcutaneous fat	487
Perirenal fat	490

*After feeding D&G wafers containing 1 per cent pristane by weight for 5 weeks. Average of 5 rats.

†Ileocecal.

The basic experimental sequence involved (1) bilateral subcutaneous injections (hind legs) totaling 0.1 ml. of either saline or 10 per cent egg albumin (Fisher A-388) in saline, or 0.2 ml. of a 1:1 emulsion of 10 per cent egg albumin and Freund's Complete Adjuvant (Difco); (2) 7 days later a second subcutaneous injection (0.1 ml., bilateral, front legs) of 10 per cent egg albumin in saline; (3) careful observation until either spontaneous death, random selection for sacrifice, or 10 days had passed after the challenge with egg albumin. The rats were maintained on their respective control or treated diet throughout this experimental period, and the investigator giving the injections did not know which rats were in which diet group.

Rats to be sacrificed were anesthetized with Diabotal, blood was withdrawn into siliconized Vacutainers, and the animals were exsanguinated. Fluid was collected from the peritoneal cavity, the viscera were examined for gross pathologic changes, and tissue samples were excised for histologic examination (routine hematoxylin and eosin and Oil Red O stains).

Circulating antibodies were sought by double immunodiffusion on trypan blue-impregnated

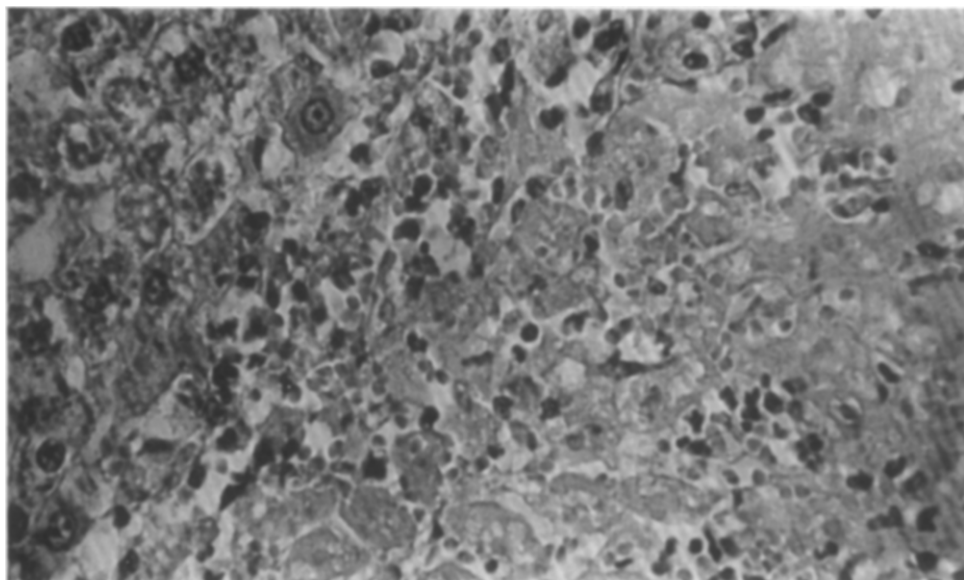


FIG. 3. Hematoxylin and eosin-stained section of liver. Note demarcation between normal-appearing and necrotic regions, and pyknotic nuclei in the latter. Group 3A. ($\times 450$.)

plates (Pentex). Serum or intraperitoneal fluid was placed in the center well and dilutions of egg albumin in saline in the wells around the periphery. In some experiments circulating egg albumin was sought with rabbit anti-egg albumin serum in the inner well and dilutions of rat serum in the outer wells. Total gamma globulins were assayed in rat serum using fluorescein-conjugated rabbit anti-rat gamma globulin (globulin fraction, Sylvania SY45-458). Fluorescence precipitated at equivalence was measured with excitation at 487 m μ and emission at 525 m μ using an Aminco-Keirs spectrophosphorimeter in the fluorescence mode.

A rough measure of tissue-associated antibody was made by measuring the decrease in fluorescence of a solution of fluorescein-conjugated egg albumin (Pentex 96-043) when treated with a homogenate of rat spleen. Aliquots of a 5 per cent homogenate of rat spleen in saline were combined with the fluorescent antigen, held at 4° overnight after 2 hours at room temperature, and centrifuged. Fluorescence remaining in the supernate was measured and corrected for the nonspecific adsorption and quenching observed using homogenized spleen from uninoculated rats.

The gross responsiveness of rats to serotonin was examined by using a Thermistemp Model 71A thermister probe accurate to 0.01° to measure rectal body temperature following a one milligram dose of serotonin as the hydrochloride in saline injected subcutaneously. The rats were awake and alert but held in restraining cages during this operation.

RESULTS AND DISCUSSION

The various treatment groups compared in the experiments discussed in this paper are summarized in Table I. Not listed in the table are over 40 rats that have been maintained on diets containing from 0.5 to 2 per cent pristane for periods of time up to 8 months and that showed no pathologic morphologic changes during this period. Table II lists typical equilibrium concentrations of pristane in some selected rat tissues under the conditions of the present experiments. These levels of hydrocarbon are insufficient to produce a histologically demonstrable "lipidosis" condition.⁹

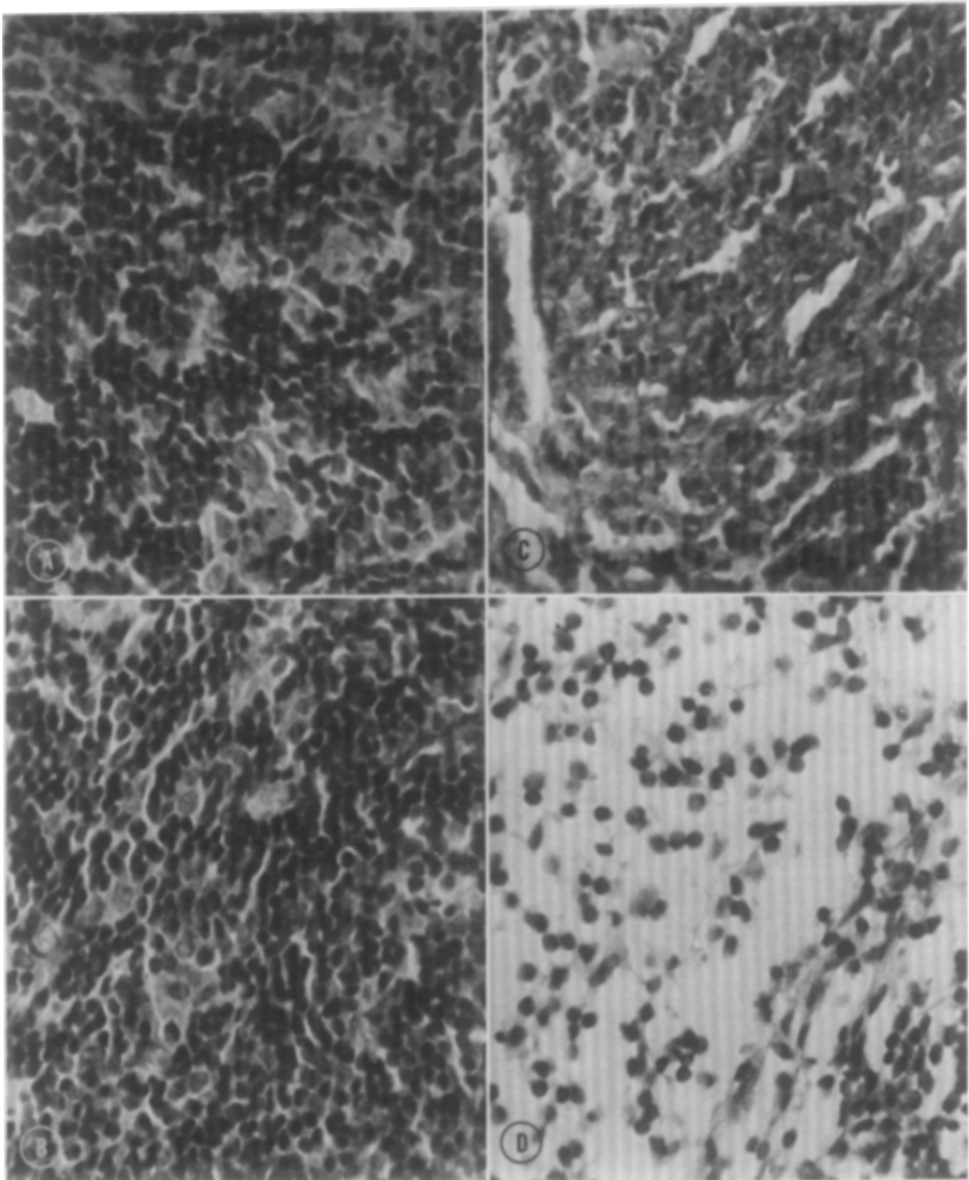


FIG. 4. Hematoxylin and eosin-stained sections of rat spleens. **A** shows a section of a spleen from group 1B (control). **B** shows a corresponding section from group 1A (pristane-fed, no albumin). No anomalies are seen. **C** shows a similar section from group 3A, pristane + 2X albumin, 48 hours after the second albumin injection. The extracellular clear areas stain with Oil Red O. **D** shows another corresponding section from group 3A, but one week after the second injection of albumin. Fatty degeneration is extensive, with only reticular cells and small lymphocytes surviving. (All $\times 450$.)

No pathologic effects were seen in any of the 19 rats fed the control diet regardless of the type of treatment with egg albumin. Rats fed the treated diet likewise showed no ill effects if they were not challenged with the second albumin injection. No pathologic effects were seen in any of the splenectomized rats.

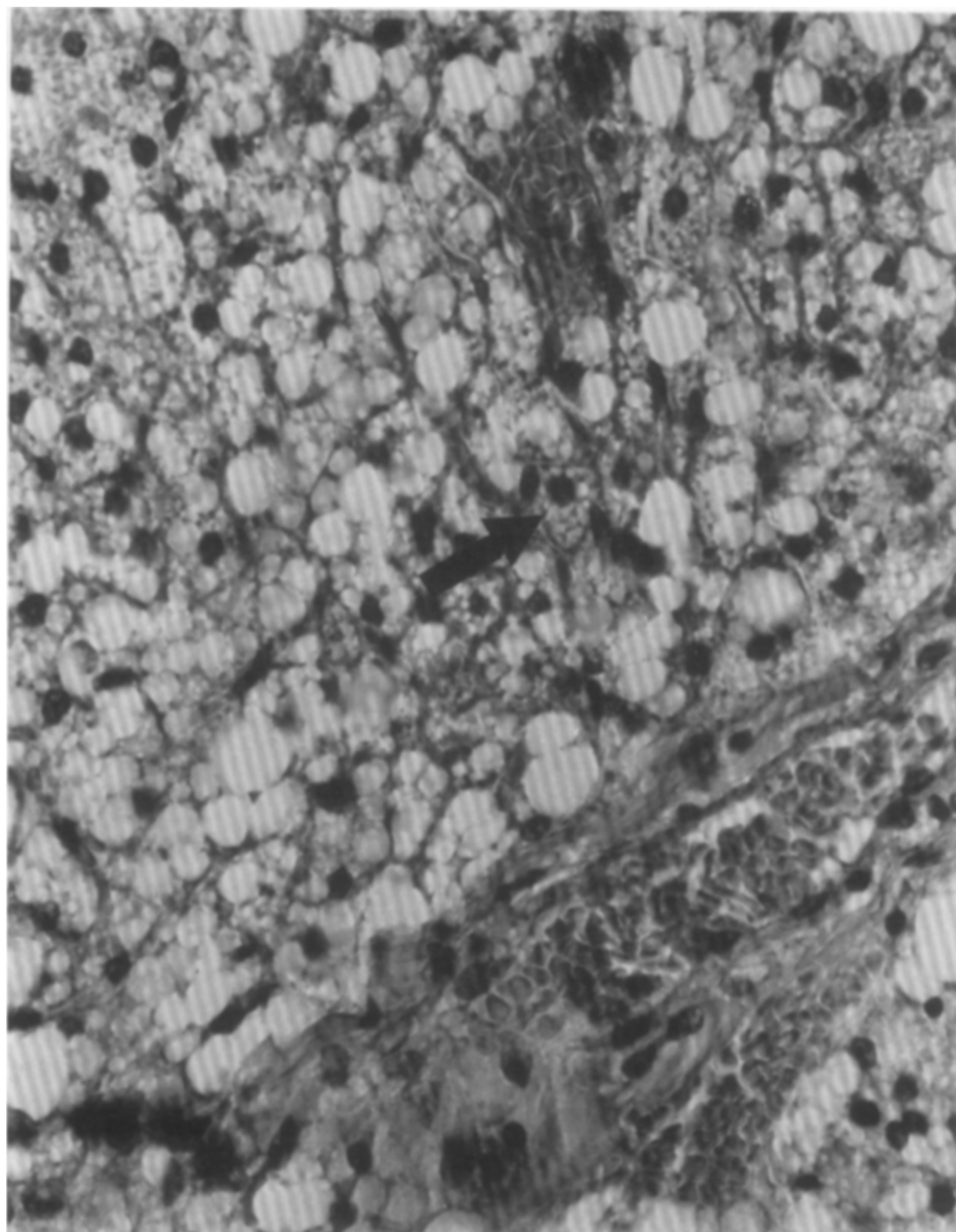


FIG. 5. Hematoxylin and eosin-stained section of celiac lymph node. From group 3A rat, 4 days after second injection of egg albumin. Clear, round areas here show up red in Oil Red O-stained sections. Oil droplets are both extracellular (large) and intracellular (small). Arrow points to oil droplets (as vacuoles in hematoxylin and eosin sections) surrounding the nucleus of a lymphocyte; others may be seen similarly located in macrophages. ($\times 950$.)

TABLE III. Binding of fluorescein-conjugated egg albumin by homogenates of rat spleen*

Treatment	N	Bound egg albumin (μ g/Gm. spleen)		P < ‡
		Total $\pm$ S.D.	Corrected†	
1. Untreated, control diet	4	19.9 $\pm$ 2.0	0	
2. 2 $\times$ albumin, control diet	6	30.8 $\pm$ 4.4	10.9	
3. 2 $\times$ albumin, pristane diet	11	24.9 $\pm$ 2.1	5.0	
3 $\bar{v}$ 1				0.002
2 $\bar{v}$ 1				0.005
3 $\bar{v}$ 2				0.005

*0.5 ml. aliquots of 20 per cent homogenates in saline were combined with 2.0 ml. aliquots of a saline solution of fluorescein-conjugated egg albumin, let stand in a cold room overnight, and centrifuged. The amount of egg albumin bound was calculated from the decrease in fluorescence intensity of the supernatant.

†Corrected for nonspecific adsorption and quenching by subtracting the values in group 1 from the others.

‡From 2 tailed t test applied to uncorrected data.

Except in the splenectomized rats, severe pathologic effects were obvious in all of the rats (26 total) fed the treated diet and given both injections of egg albumin (groups 3A and 4A). There were no obvious differences in types of responses between groups 3 and 4. Thus they will be considered together in the discussion that follows.

The rats in groups 3A and 4A showed different types of pathologic responses, depending primarily on how long they survived after the second injection of albumin. Five died spontaneously, 3 in less than one hour and 2 about 48 hours after the albumin injections. The former 3 died apparently from extensive hemorrhaging in the lungs and showed no other obvious abnormalities. The latter 2 hemorrhaged extensively into the lower small intestine and cecum, with slight bleeding in the lungs and pancreas (Fig. 1).

A total of 9 pristane-fed rats bled extensively into the intestines, stomach, or bladder. Nine rats (not necessarily the same 9) hemorrhaged to some extent in the lungs—but a total of 20 out of the 26 group 3A plus 4A rats showed conspicuous bleeding in the pancreas, and this appeared to be one of the most distinctive early responses to the treatment.

Of the 21 group 3A plus 4A rats surviving for at least 48 hours beyond the second albumin treatment, 15 showed massive visceral adhesions. Most commonly, the liver, spleen, pancreas, stomach, and diaphragm were fused together. Often the spleen was deeply embedded in an enlarged liver. Histologic examination of these adhesions showed a reticular Oil Red O staining interphase between the adhering organs. All of the 26 rats in groups 3A and 4A, by 48 hours after the last injection of egg albumin, showed either the hemorrhaging, the adhesions, or both.

Between 2 and 7 days after the albumin treatment, surviving rats in groups 3A and 4A showed a variety of pathologic effects other than those already described. Fibrous intestinal tumors (Fig. 2), hemorrhaging around the seminal vesicles, and highly enlarged lymph nodes were commonly seen. Liver necrosis (Fig. 3), fatty degeneration of the spleen leading to enlargement and loss of red

TABLE IV. Body temperature lowering by subcutaneously injected serotonin*

Rat diet, 5 weeks	N	Min. for 1° drop	Total drop (°)
Untreated	3	34 ± 6	1.6 ± 0.3
Pristane-treated	4	41 ± 5	1.4 ± 0.4

*One milligram serotonin in 0.1 ml. saline, 385 ± 25 Gm. rats. Each value is the average of 2 determinations on each rat, 24 hours apart.

pulp (Fig. 4), and fatty infiltration of the lymph nodes (Fig. 5) were prevalent by one week after treatment.

We could not detect circulating antibodies against egg albumin in serum or intraperitoneal fluid from any of the inoculated rats by the double-diffusion technique. However, the lower limit of sensitivity, determined using rabbit anti-ovalbumin, was 30 to 40 μ g of antibody protein per milliliter. Total serum globulins were at twice the level in the groups 3A and 4A rats of that in the groups 3B and 4B rats 4 days after the second injection of egg albumin. Egg albumin itself was clearly detected 48 hours after the second injection in serum from rats in groups 3A and 4A, but not in serum from rats in groups 3B and 4B.

Table III summarizes the results of assays for splenic anti-egg albumin carried out 4 days after the second albumin injection. The pristane-treated rats apparently accumulated or retained only half as much splenic anti-egg albumin activity as did control rats. This could reflect greater access of the subcutaneously injected albumin to the spleen in the pristane-fed rats, resulting in neutralization of a larger number of binding sites. Alternatively, the pristane-treated rats may have simply produced less specific, spleen-associated anti-egg albumin than the controls. The smaller number of rats in group 2 precludes drawing any conclusions from their apparent resistance, but it is certainly suggestive to note that the splenectomized animals were the only pristane-fed animals escaping injury after 2 injections of egg albumin in these experiments.

Table IV briefly summarizes the responsiveness of rats to subcutaneously injected serotonin as indicated by their body temperatures. The pristane diet had no statistically significant effect on apparent sensitivity to serotonin, although a slight trend toward decreased sensitivity was suggested for the treated group. We also failed to observe any effect of the pristane diet on responsiveness to subcutaneously injected histamine in this assay, but the refractory status of rats toward histamine, even in control animals, resulted in changes in body temperature that were too slight and transient to be useful.

The only effect we were able to observe after the pristane-fed rats were given a single treatment with egg albumin was the almost total disappearance of mast cells from the intraperitoneal fluid. This did not occur in rats on the control diet.

The phenomena reported here were unexpected at the time the experiments were planned and therefore have not been studied in a systematic and thorough manner. The egg albumin generally used as antigen was apparently prepared by ammonium sulfate precipitation and was only about 90 per cent pure ovalbumin, with some conalbumin visible on electrophoresis. However, it was extremely low in ovomucoid. We have made some preliminary studies, not reported in detail here, using other antigen preparations: Sigma Grade V egg

albumin (99 per cent pure) appears to be as effective as the Fisher product, but various preparations of bovine serum albumin were ineffective.

The pathologic effects reported here apparently required two conditions: (1) a tissue "burden" of hydrocarbon or its metabolites, or some predisposition resulting from there having been such a burden; and (2) at least two exposures to a strong antigen. Neither treatment alone produces any detectable pathologic response under the conditions used here. Our simple exploratory experiments do not suggest any mechanism by which dietary pristane predisposes rats to the egg protein-triggered pathologic responses, but some of the results suggest lines of future study. The negative responses of splenectomized rats and decreased splenic anti-egg albumin in pristane-fed, albumin-treated rats suggest, but because of the small number of animals do not prove, a significant involvement of the spleen. The only "predisposing" effect we were able to observe was the disappearance of peritoneal mast cells in pristane-fed rats following the initial injection of egg albumin, and this phenomenon may also deserve further study. We are presently studying the metabolism of isoprenoid hydrocarbons in rats in the hope that some light will be shed on the adverse biologic activity of pristane in this context.

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