PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 147, 775-779 (1974)

Effect of Freund's Adjuvant on Blood Amylase, Fecal Trypsin, and Hematocrit Levels in Laboratory Mice (38436)

O. H. PIVETTA[1] AND N. KALISS[2]

The Jackson Laboratory, Bar Harbor, Maine 04609[3]

High blood amylase levels in humans may be indicative of a variety of disorders: acute pancreatitis, perforated peptic ulcer, acute nonperforated cholecystitis, uremia, ectopic pregnancies, etc. (1–3). Morphine and codeine injections have been shown to elevate serum amylase levels (4). In light of the observation by Gray *et al.* (5), it is likely that an acute pancreatitis may underly the increased amylase levels in most of these dyscrasias.

In mice and rats the pancreas apparently supplies significant amounts of serum amylase. This is suggested by decreases in pancreatic and serum amylase activity accompanying fasting, partial pancreatectomy, and parenteral administration of ethionine (6).

For our studies on cystic fibrosis in the laboratory mouse we were interested in the possibility of producing an "autoimmune" pancreatitis by injection of alloxan-treated pancreatic tissue incorporated in Freund's complete adjuvant. Alterations in normal blood amylase and fecal trypsin levels were to be indicators of the condition. We were not able to ascribe a pancreatitis as specifically associated with the tissue-adjuvant treatment, but we did note, however, that certain inbred strains of mice given adjuvant alone exhibited alterations in enzyme levels that might be attributable to pancreatitis. The present report presents experiments designed to define the role of the adjuvant and to establish whether the animal's response is genetically controlled.

Materials and Methods. Mice were females (five mice per experimental group) from The Jackson Laboratory's inbred strains AKR/J, B10.D2/nSnJ, C57BL/10J (abbreviated B10), DBA/2J (abbreviated D2), and 129/J. B10 (*H-2*^{b}) and B10.D2/n (*H-2*^{d}) are congenic strains; the progenitor strains for B10.D2/n are B10 and D2 (*H-2*^{d}) (7). B10 and B10.D2/n presumably differ genetically only at the H-2 histocompatability region. Mice of all strains except B10.D2/n were 10–11 weeks old on entry in the experiments; B10.D2/n mice were 14–19 weeks old. The animals had free access to water and a pelleted feed (Old Guilford).

With the exception of the AKR strain, the choice of strains was predicated on their being possible candidates for producing an autoimmune pancreatitis. A preliminary assay of fecal trypsin content showed D2 with high normal levels and B10 with the lowest levels. On this basis, these two strains and B10.D2 were selected for survey. 129/J was chosen because it is a strong immune responder to a variety of immunogens (N. Kaliss, unpublished data). AKR was selected since it had been shown, in contrast with the other strains, to produce exceptionally large quantities of ascitic fluid after receiving Freund's complete adjuvant ip (B. B. Jacobs and N. Kaliss, to be published).

Experimental treatments. The materials were administered in a single ip injection of 0.2 ml per mouse. Freund's complete (FCA) or incomplete adjuvant (FA) (without Mycobacterium) were from Difco (Detroit, MI). Heat-killed and freeze-dried Bacillus Calmette–Guerin (BCG) (*M. bovis*) (Trudeau Institute, Saranac Lake, NY) were suspended in 0.15 *M* NaCl (0.5

[1] Supported by NIH Research Grant No. AM 15587 from the National Institute of Arthritis, Metabolism, and Digestive Diseases and by a grant from the National Cystic Fibrosis Research Foundation. Present address: Centro de Genetica Médica, Buenos Aires, Argentina.

[2] Supported by NIH Research Grant No. CA 01594 and NIH Grant No. SO1 RR05545 from the National Cancer Institute, and by the Eva Gebhard-Gourgaud Foundation.

[3] The Jackson Laboratory is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

mg/ml, w/v). The quantity of bacteria thus injected (0.1 mg) is equivalent with that of the *M. butyricum* incorporated in 0.2 ml of FCA. "Saline" injections were of 0.15 *M* NaCl. To evaluate a possible effect of the route of administration, groups of B10.D2 females received 0.2 ml per mouse of either FCA or saline sc.

Determination of enzyme activity. Plasma amylase was assayed in blood (obtained from a sinus in the inner canthus of the eye) with Amylchrome (Roche Diagnostics Division, Hoffman-LaRoche, Nutley, NJ). Amylchrome's chromogenic substrate is acted upon in direct proportion to amylase levels; enzyme activity is expressed in dye units/100 ml plasma (8, 9). Trypsin activity was determined in 10% feces suspensions (v/v) by the gelatin tube test for fecal trypsin (10).

Hematocrits were done by a standard micromethod (11) for blood taken from the sinus in the inner canthus of the eye.

Histology. Pancreas, salivary glands, spleen, kidney, and liver were taken from mice asphyxiated by CO_2 7 weeks after they had received the various treatments. The organs were fixed in a modified Bouin's fluid (12), embedded in paraffin, sectioned at 8 μm, and stained with eosin and hematoxylin.

Results. Blood amylase levels. The data are given in Table I. Not included are strains 129 and AKR since no significant differences between the groups receiving the different treatments occurred in these strains. Significantly elevated enzyme levels first appeared 2 weeks after injection of either FCA or FA, most markedly so in the B10.D2 and B10 mice, and persisted for 6 weeks (the time of the last observation). In the affected groups it is clear that the causative agent or agents are either the Bayol F mineral oil or Arlacel (mannide monooleate) constitutents, or both, of FCA and FA. BCG, the third component of FCA, had no effect.

Fecal trypsin levels. The data are given in Table II. The B10.D2, D2, and B10 mice are the same animals giving the amylase data of Table I. Not included are strains 129 and AKR; these did not exhibit enzyme alterations with FCA and FA treatment. Significantly lower enzyme levels appeared only in B10.D2 mice at 2 and 4 weeks after FCA injection and at week 2 after FA. By week 6 the levels approximated those of the saline controls.

Hematocrits. Determinations were made because of the observation that bloods of mice given adjuvant appeared "thin." Assays were made before treatment and at 1, 2, 4, and 6 weeks after treatment. A significant drop in values appeared as early as 1 week posttreatment and persisted thereafter. Data for some of the bleedings are listed in Table III.

Effect of sc injection. Table IV presents data for B10.D2 females given 0.2 ml each of either FCA or saline sc. There were no significant

TABLE I. Plasma Amylase Activity for Mice Receiving Injections ip.

Mouse strain	Material injected[a]	Amylase values (mean ± SE) in dye units/100 ml[b]			
		Before injection	After injection		
			Week 2	Week 4	Week 6
B10.D2/n	FCA	1931 ± 498	7637 ± 1020[c]	5365 ± 708[c]	6227 ± 876[c]
	FA	2229 ± 59	9931 ± 561[c]	6589 ± 467[c]	5228 ± 274[c]
	BCG	2332 ± 45	2649 ± 130	2525 ± 133	2599 ± 170
	Saline	2545 ± 118	2599 ± 189	2430 ± 80	2745 ± 89
B10	FCA	3022 ± 205	5545 ± 856[d]	4809 ± 957[c]	8461 ± 2030
	Saline	3281 ± 369	2005 ± 73	2587 ± 108	3781 ± 432
D2	FCA	2718 ± 89	3143 ± 178[d]	3095 ± 214	2183 ± 169
	Saline	2716 ± 41	2357 ± 119	2828 ± 63	2299 ± 168

[a] FCA = Freund's complete adjuvant; FA = incomplete adjuvant; saline = 0.15 *M* NaCl.

[b] Tests for statistical significance are versus the "Saline" group, by analysis of variance; five females per group. Each B10.D2 and B10 mouse was assayed for both amylase and trypsin (see Table II).

[c] $P < 0.05$.

[d] $P < 0.01$.

TABLE II. Fecal Trypsin Values for Mice Receiving Injections ip.

Mouse strain	Material injected	Trypsin activity (Z)[a] Before injection	After injection Week 2	Week 4	Week 6
B10.D2/n	FCA	1.8	0.4[b]	0.2[b]	1.0
	FA	1.8	0.2[c]	2.0	1.6
	BCG	1.4	1.4	2.0	1.0
	Saline	2.0	2.0	1.8	1.2
B10	FCA	0.8	0.4	0.8	1.2
	Saline	0.8	1.2	1.0	0.4
D2	FCA	1.8	2.0	2.0	1.2
	Saline	2.0	1.8	1.6	2.0

[a] $Z = 2X_1 + X_2 + OX_3/y$ where X_1 = number of fecal samples positive (+); X_2 = number of marginally positive (±) samples; X_3 = number of negative (−) samples; y = number of mice tested per group (five). Each B10.D2 and B10 mouse was assayed for both trypsin and amylase (see Table I).

[b] $P < 0.05$ vs saline, by chi-square test.

[c] $P < 0.01$ vs saline

differences between the FCA- or saline-treated groups. Each mouse was assayed for amylase and trypsin levels and for hematocrits.

Gross pathology. Three mice of each treatment group of the B10, B10.D2, and D2 strains, and two mice of each group of strains 129 and AKR were autopsied 7 weeks after treatment. The following descriptions are for the mice given FCA (the FA-treated mice presented a similar picture) unless otherwise stated. Comparisons are with the groups given saline.

Two of the three B10 mice developed ascitic fluid. The peritoneal organs in all three animals were encapsulated in a heavy fibrotic layer of tissue. (The morphology of this layer, a lipogranulomatous tissue reaction, is described in detail by Potter and Robertson (13).) The pancreas was unidentifiable in the fibrous overgrowth. Spleens were enlarged two to four times. Livers were somewhat hypertrophied and hard. Salivary glands were smaller and more compact in the two mice with ascites fluid than in the third animal. These glands contrasted with the lighter colored and softer glands in the mice given saline. The D2 mice presented a picture identical with that of the B10 mice.

TABLE III. Hematocrit Values for Mice Receiving Injections ip.

Mouse strain	Material injected	Hematocrits (mean ± SE) Before injection[a]	After injection Week 2	Week 6
129	FCA	55.70 ± 0.49	45.50 ± 2.22[b]	45.00 ± 2.81[b]
	Saline	56.60 ± 0.48	53.10 ± 0.70	54.20 ± 0.58
B10.D2/n	FCA	52.20 ± 0.20	40.90 ± 0.75[b]	47.60 ± 0.40[b]
	FA	53.20 ± 0.49	45.90 ± 0.56[b]	49.00 ± 0.42[c]
	Saline	53.10 ± 1.23	50.50 ± 0.39	51.80 ± 0.49
B10	FCA	ND	39.70 ± 0.46[c]	43.20 ± 1.25[c]
	Saline	ND	44.50 ± 1.02	49.30 ± 1.24
D2	FCA	48.20 ± 0.41	45.30 ± 0.80[b]	48.10 ± 0.51[c]
	Saline	48.40 ± 0.48	48.40 ± 0.93	50.40 ± 0.40

[a] ND, not done.

[b] $P < 0.05$ by analysis of variance.

[c] $P < 0.01$.

TABLE IV. Amylase, Hematocrit, and Trypsin Values for B10.D2 Mice Receiving Injections sc[a].

Parameter	Material injected	Values before injection[a]	Values after injection[a]		
			Week 2	Week 4	Week 6
Amylase activity	FCA	2891 ± 224	2301 ± 139	2812 ± 227	2688 ± 208
	Saline	2853 ± 101	2166 ± 43	2898 ± 100	2736 ± 97
Hematocrit	FCA	50.90 ± 0.51	50.60 ± 0.58	49.50 ± 0.59	49.80 ± 0.78
	Saline	52.00 ± 0.52	50.50 ± 0.59	50.00 ± 0.35	52.20 ± 0.97
Trypsin level	FCA	1.8	2.0	1.2	ND
	Saline	1.8	2.0	1.4	ND

[a] Values are means ± SE for amylase activity and hematocrit for two groups of five females each. Trypsin levels are *Z*-values (see footnote *a*, Table II). Each mouse was assayed for the three parameters.

The B10.D2 mice exhibited the fibrotic overgrowth but the pancreases were identifiable and appeared hypertrophic. The spleens were somewhat enlarged in two of the three mice. The livers were normal. The salivary glands were reddish and somewhat compact. The mice given BCG appeared normal in all respects.

The two AKR mice did not have as heavy a fibrotic overgrowth as did the other strains. One of the mice developed ascites fluid in large volume; this mouse had a fibrotic pancreas, a somewhat enlarged and compact liver, and an enlarged spleen. One of the two 129 mice presented a similar picture.

Histology. (We thank Dr. Hans Meier for the histological analysis.) The following descriptions for FCA-treated B10.D2 animals hold in general for the four other strains of mice given FCA. The spleen and a submaxillary lymph node (adjacent to the submaxillary gland) exhibited a lymphoreticular hyperplasia. The pancreas was edematous and hyperemic and there was some lobular destruction and a lipogranulomatous reaction. The hilus of the spleen exhibited similar complications. The liver had a peripheral lymphocytic infiltration and scattered foci with a granulomatous reaction. In contrast, there was a lymphoreticular but no granulomatous reaction in the mice given BCG.

The most severe reactions were in a 129 mouse, but its pancreas was somewhat less involved than in B10.D2 animals and the liver was quite cellular. Tissues of the mice given saline appeared normal in all strains of mice.

Discussion. Our data show that the oil component(s) of FCA is the initiator of the fibrotic tissue reaction in mice given FCA or FA; BCG alone had no adverse effect. The irritant may be the mineral oil (Bayol F). It has been demonstrated in strain BALB/c mice to be as effective by itself as were FA plus Staphylococcus in inducing a peritoneal lipogranulomatous tissue reaction and the subsequent myelomas which apparently originated in the reactive tissue (13).

The increase in amylase and decrease in trypsin levels reflects a local effect of the adjuvant since the alterations appeared in the animals receiving ip injections and not in those treated sc. What tissues or organs are involved is not evident from our experiments. It has been assumed by other workers that the pancreas and salivary glands are the sole source of serum and extracellular amylase. Evidence is accumulating, however, that in addition, an extrapancreatic and extrasalivary gland source (or sources) maintains the serum levels. Calculations of tissue-to-serum amylase ratios and extracellular fluid levels indicate that liver in rat, mouse, guinea pig, and dog, and muscle in mouse and guinea pig contain intracellular amylase (14).

Our finding that a pancreatitis exists in all the FCA-treated strains, including those with no altered enzyme levels, would argue against damage to the pancreas as responsible for the changes in B10.D2/n mice. The identity of findings for the gross and microscopic pathology for FCA-treated mice in all the five strains further speaks against damage per se to other peritoneal organs or the lipogranulomatous reaction as underlying the alterations in the B10 and B10.D2 mice. The nature of the functional changes responsible for the shift in enzyme levels cannot be determined from our data. The significant decreases in hematocrits in the B10, B10.D2/n, D2, and 129 strains, however, probably are attributable to tissue damage.

It appears that whether or not physiological alterations, expressed in the changed enzyme

levels, are produced by the irritant action of Freund's adjuvant is genetically controlled. The genetic aspects are emphasized by the much higher reactivity of the B10 and B10.D2/n strains as compared with the D2 strain. The B10.D2 strain was produced by successive backcrosses to the B10 strain of descendants from an original (B10 × D2)F_1 progeny, with genetic disparity between B10 and B10.D2 being maintained only for the H-2 histocompatibility region (7). B10.D2's "amylase susceptibility" thus would be derived from the B10 strain. Strain (and, therefore, genetic) differences in reaction to ip injection of mineral oil also hold for the induction of myelomas; thus far, only the inbred BALB/c strain appears to be peculiarly susceptible (13), and the C3H strain to a lesser extent.

Summary. Ip injections of complete or incomplete Freund's adjuvant caused an elevation in blood amylase and a reduction in fecal trypsin levels in three inbred strains of mice; they were without effect in two inbred strains. Hematocrits were significantly reduced in all the strains so treated. Injections ip of Bacillus–Calmette–Guérin alone were without effect. Administration sc of the adjuvants was without effect. Alterations were most marked in two congenic strains and persisted for at least 6 weeks after treatment. Peritoneal organs of the mice given adjuvant were encapsulated in a heavy fibrotic layer of tissue. Histologically, in all strains the pancreas was edematous and hyperemic and there was some lobular destruction. The effects of the adjuvants are attributed to direct irritant action of the oil component of the adjuvant on the peritoneal organs. Whether or not amylase and trypsin levels will be altered in consequence appears to be genetically controlled.

We thank Diane Gaudreau and Stephen H. Langley for excellent technical assistance.

1. Frankel, S., *in* "Grandwohl's Clinical Laboratory Methods and Diagnosis" (S. Frankel, S. Reitman, and A. C. Sonnenwirth, eds.), p. 111. C. V. Mosby, St. Louis, MO (1963).

2. Bailey, G. L., Katz, A. I., Hampers, C. L., and Merrill, J. P., J. Amer. Med. Ass. **213,** 2263 (1970).

3. Flege, J. B., Jr., Arch. Surg. **92,** 397 (1966).

4. Gross, J. B., Vomfort, M. W., Methieson, D. R., and Power, M. H., Proc. Staff Meet. Mayo Clinic **26,** 81 (1951).

5. Gray, S. H., Probstein, J. G., and Heifetz, C. J., Arch. Int. Med. **67,** 805 (1941).

6. Wiberg, C. S., and Tuba, J., Can. J. Biochem. Physiol. **33,** 817 (1955).

7. Snell, G. D., and Stimpfling, J. H., *in* "Biology of the Laboratory Mouse" (E. L. Green, ed.), Ed. 2, p. 457. McGraw-Hill, New York (1966).

8. Klein, B., Foreman, J. A., and Searcy, R. L., Clin. Chem. **16,** 32 (1970).

9. Take, S., Berk, J. E., and Fridhandler, L., Clin. Chem. Acta **26,** 533 (1969).

10. Schwachman, H., Patterson, P. R., and Laguna, J., *in* "Grandwohl's Clinical Laboratory Methods and Diagnosis" (S. Frankel, S. Reitman, and A. C. Sonnenwirth, eds.), p. 1960. C. V. Mosby, St. Louis, MO (1963).

11. Simmons, A., *in* "Technical Hematology" (A. Simmons, ed.), p. 54. Lippincott, Philadelphia (1968).

12. Lillie, R. D., "Histopathologic Technic and Practical Histochemistry," ed. 3, p. 39. McGraw-Hill, New York (1965).

13. Potter, M., and Robertson, C. L., J. Nat. Cancer Inst. **25,** 847 (1960).

14. MacGeachin, R. L., Gleason, J. R., and Adams, M. R., Arch. Biochem. Biophys. **75,** 403 (1958).

Received June 18, 1974. P.S.E.B.M., 1974, Vol. 147.