

- 5 CEPPELLINI, R.: Physiological genetics of human blood factors; in *Biochemistry of human genetics*, Ciba Foundation Symposium, Boston (Little, Brown, Boston 1959).
- 6 COHEN, S. and MILSTEIN, C.: Structure and biological properties of immunoglobulins. *Adv. Immunol.* 7: 1 (1967).
- 7 DRAY, S. and YOUNG, G. O.: Differences in the antigenic components of sera of individual rabbits as shown by induced isoprecipitins. *J. Immunol.* 81: 142 (1958).
- 8 GAHNE, B.: Isoantibodies against slow α_2 -globulin in cattle. *Hereditas* 51: 375 (1964).
- 9 GRABAR, P. and BURTIN, P. (ed.): *Immuno-electrophoretic analysis*. (Elsevier, Amsterdam 1964).
- 10 GIBLETT, E. R.: *Genetic markers in human blood* (Blackwell, Oxford/Edinburgh 1969).
- 11 KASUKAWA, R.; YOSHIDA, T., and MILGROM, F.: An isoprecipitin distinguishing polymorphism of human serum α_1 -globulin. *Int. Arch. Allergy* 36: 347 (1969).
- 12 KNIGHT, K. L. and DRAY, S.: Identification and genetic control of two rabbit α_2 -macroglobulin allotypes. *Biochemistry* 7: 1165 (1968).
- 13 LEIKOLA, J.; FUDENBERG, H. H.; KASUKAWA, R., and MILGROM, F.: A new genetic polymorphism of human serum. *Amer. J. hum. Genet.* (in press).
- 14 LICHTER, E. A.: Discussion paper. Isoprecipitins to α_2 -macroglobulins in chimpanzees. *Ann. N. Y. Acad. Sci.* 162: 202 (1969).
- 15 OUDIN, J.: L'allotypie de certains antigènes protéidiques du sérum. *C. R. Acad. Sci.* 242: 2606 (1956).
- 16 RAPACZ, J.; HASLER, J., and POPE, A. L.: Lipoprotein serum antigen in sheep; in *Proc. 12th Int. Conf. Animal Blood Groups and Biochemical Polymorphisms*, Budapest, Hungary, 1970.
- 17 SCHEIDEGGER, J. J.: Une micro-méthode immunoélectrophorétique. *Int. Arch. Allergy* 7: 121 (1955).
- 18 SNYDER, L. H.: Inherited taste deficiency. *Science* 74: 151 (1931).
- 19 STONE, W. H.: Immunogenetics of type-specific antigens in animals; in *GREENWALDT Advances in immunogenetics* (Lippincott, Philadelphia 1967).
- 20 STORMONT, C.: Linked genes, Pseudoalleles and blood groups. *Amer. Naturalist* 89: 105 (1955).
- 21 WIEME, R. J.: Characterizing reactions applied to agar gel electrophoresis; in *Agar gel electrophoresis* (Elsevier, Amsterdam 1965).
- 22 WIEMER, H. E. and TOGUCHI, R.: Chemical, electrophoretic and immunochemical studies of the seromucoid fraction of serum. A species comparison. *Comp. Biochem. Physiol.* 28: 887 (1969).

Authors' address: Dr. J. A. BASH and Dr. F. MILGROM, Department of Microbiology, School of Medicine, State University of New York, Buffalo, NY 14214 (USA)

Int. Arch. Allergy 42: 215-224 (1972)

The Effect of Laundry Detergent Containing *B. subtilis* Enzyme in Producing Sensitization Reactions in Guinea Pigs

L. PERELMUTTER, A. LAVALLÉE and G. S. WIBERG

Food and Drug Directorate, Ottawa, Ontario

Abstract. Guinea pigs exposed to laundry detergents containing *B. subtilis* enzymes (alcalase) under home use conditions, i.e. by inhalation, abraded skin or topical application and oral ingestion, produced sensitization reactions. The jejunum smooth muscles of these animals contracted in the presence of alcalase and the sera caused morphological changes of rat mast cells in the presence of alcalase. It was found that long periods (50-60 days) of chronic exposure was necessary to produce sensitization. The relevance of these results to sensitization of consumers to *B. subtilis* enzymes in laundry detergent is discussed.

A rather high frequency of allergic respiratory disturbances has been noted in factory workers handling detergents containing *B. subtilis* enzymes [1-5]. Recently, several investigations [6-10], have shown that some sensitization occurred in populations (consumers) not exposed to high concentrations of enzyme dust. Evidence for sensitization of the enzymes was based on clinical histories, intradermal skin-tests and provocation reactions. It was concluded that type 1 hypersensitivity reactions involving IgE-type skin-sensitizing antibodies were mainly responsible for such allergic manifestations. GRIFFITH *et al.* [11] found that the *B. subtilis* enzymes were not effective in causing contact sensitivities in a large group of consumer population nor in guinea pigs and rabbits. In addition, the acute oral administration of detergents containing *B. subtilis* enzymes to rats displayed a low order of oral toxicity.

As yet, no large scale studies in consumers have been reported and the level of sensitization of consumers to *B. subtilis* enzymes is un-

Received: September 24, 1971.

known. Furthermore, no attempt has been made to determine whether these enzymes can cause sensitization in animals exposed to the detergent enzyme under simulated household conditions. The purpose of the present study, was to simulate conditions of consumer use of enzyme-containing detergents in the guinea pigs in order to establish whether exposure of these animals to an alcalase-containing detergent by inhalation, topical and abraded skin application and oral ingestion routes would yield allergic manifestations. Examination of these animals on long-term exposure revealed sensitization of the jejunum and the presence of circulating antibodies to alcalase as determined by the rat mast cell technique [12-14].

Materials and Methods

B. subtilis Enzymes and Laundry Detergent

A commercial preparation of alcalase was used in this study to immunize and test for antigens by Schultz-Dale, passive cutaneous and rat mast cell test. The preparations were obtained from Proctor and Gamble Co. of Canada. A laundry detergent, known to contain alcalase was also employed.

Animals

Albino female guinea pigs, weighing 300-450 g, were used for exposure experiments outlined below. These animals were housed in standard accommodation and fed a commercial guinea pig ration and lettuce. The jejunum from these animals were used for the Schultz-Dale experiments and sera from these animals were analyzed by the passive cutaneous anaphylaxis and rat mast cell techniques described below.

Exposure Conditions

1. Injection

Two groups of 6 guinea pigs each were separately injected by the i.p. route with either enzyme detergent or alcalase. One ml of aqueous detergent solution (5 mg/ml) or alcalase (1 mg/ml) plus 1 ml of Freund's complete adjuvant was employed for immunization. This mixture was injected weekly for 3 weeks and the animals tested for sensitization at least 9 weeks after the last injection.

2. Household Simulation Conditions

(a) *Inhalation*. Six guinea pigs were placed in an open plastic mouse cage (10×8×4 in) which in turn was put at one end of a cardboard box (2×1×1 ft). Approximately 250 ml of dry laundry detergent was poured through an opening at the top of the box so as to avoid direct contact of the detergent with the guinea pigs. The opening was closed and the guinea pigs were exposed to the detergent dust for 30 min. This procedure was performed twice daily, for 5 consecutive

days, weekly, for various periods of exposure. The test animals were examined for sensitization as described below.

(b) *Oral ingestion*. Two ml of detergent (50 mg/ml) were sprinkled on the lettuce of 6 guinea pigs once a day for 5 consecutive days, weekly. The guinea pigs were then removed from the test at various times up to 64 days of oral exposure and tested accordingly.

(c) *Intact skin*. A 1-mg/ml solution of the detergent was applied with a brush on the backs of 4 guinea pigs once a day 5 times weekly. Animals were removed from the test at various times up to 59 days.

(d) *Abraded skin*. Six guinea pigs were exposed to the enzyme containing detergent in a similar manner as described in 2c except the skin of the animals were abraded once a week prior to exposure with fine sandpaper.

Criteria for Examining Animals for Sensitization

Schultz-Dale Technique

The jejunums from treated or control (normal) guinea pigs were tested for muscular contraction in the presence of alcalase using the classical Schultz-Dale technique. Contraction of jejunum were measured with a fraction-free transducer (J. Degrott Assoc., Toronto, Ont.) connected to a linear recorder. Each jejunum strip was tested for contraction using a histamine standard (10^{-4} g/ml) and then challenged with increasing concentrations of alcalase in saline solution ranging from 10^{-6} , 10^{-4} , 10^{-3} , 10^{-2} g/ml. Aliquots of 0.1 ml of antigen solutions were introduced into the 10-ml Schultz-Dale bath maintained at 37 °C. The jejunum strips were checked for contractions with histamine after antigen challenge to ensure tissue viability. The results of the Schultz-Dale responses were reported as histamine equivalents.

Rat Mast Cell Technique [12, 14]

Sera from guinea pigs on treatment and those from normal animals were examined for their ability to produce specific cytopathic effects in rat peritoneal mast cells on challenge with alcalase. The same procedures for the preparation of the mast cell suspension and test with human allergic serum and antigen have been used in the present study and has been described previously [12, 13]. Briefly, equal volumes of 0.05 ml of guinea pig serum, alcalase solution and mast cell suspension were incubated at 37 °C for 3 min. A 2-percent glutaraldehyde solution in 0.2M sodium phosphate buffer, pH 7.3, was added to the cell mixture. After 60 min at 4 °C, the cell mixture was spun in a PR2 International centrifuge at 300 g for 5 min at 4 °C. The bulk of the supernatant was discarded and the cellular pellet was resuspended in a minimum amount of this supernatant solution (0.1 ml). The cells were placed on a microscope slide and dyed with Wright stain as previously described [14]. A differential count of about 150 mast cells was made over several widely-separated optic fields and the results expressed as the percent of morphologically altered cells less serum control values.

Passive Cutaneous Anaphylaxis (PCA)

The sera from treated and control guinea pigs were examined for their ability to elicit PCA reactions on the skin of normal guinea pigs on aliquot of 0.1 ml of undiluted sera or diluted 1:5, 1:10, 1:20 and 1:40 in saline solution was injected i.d. into separate sites. After 24 h, 0.4 ml of solution containing 0.1 ml alcalase (1–5 mg/ml) and 0.3 ml of Niagara sky blue (5%) solution was injected i.p. Bluening reactions were observed 15–20 min after the i.p. injection.

Results

In order to determine if alcalase itself or alcalase in the detergent was effective in producing sensitizing antibodies in the guinea pig, 6 guinea pigs each were injected i.p. with alcalase and detergent-containing alcalase. It can be seen from table I and figure 1 that the jejunum of the injected animals contracted in the presence of alcalase. In addition, sera from these two groups of animals gave positive PCA reactions and were capable of eliciting morphological changes in rat mast cells in the presence of alcalase. It was interesting to note that the guinea pigs elicited marked sensitization 50–70 days after their last injection indicating the presence of long-lasting sensitizing antibodies.

Once it was established that alcalase alone or in detergent could sensitize guinea pigs by the injection route and that the Schultz-Dale, PCA and rat mast cell techniques could detect antibodies, other test animals were then exposed to the alcalase containing detergent by simulating

Table I. Responses of guinea pigs injected with laundry detergent or alcalase

Guinea pig No.	Antigen	Time after last injection, days	S-D	PCA titer	RMCT
1	detergent	57	+++	40	++
2	detergent	57	ND	40	++
3	detergent	66	+++	10	+
4	detergent	66	ND	10	ND
5	alcalase	57	+++	40	+++
6	alcalase	57	ND	10	++
7	alcalase	70	+++	40	+++
8	alcalase	70	ND	20	+++

S-D, Schultz-Dale; RMCT, rat mast cell technique; ND, not done.

Table II. Schultz-Dale responses of guinea pigs exposed by inhalation of laundry detergent

Guinea pig No.	Length of exposure days	Responses ¹ alcalase concentration in bath, g/ml			
		10 ⁻⁸	10 ⁻⁷	10 ⁻⁵	10 ⁻³
1	38	—	—	—	—
2	39	—	—	—	—
3	52	—	—	—	—
4	58	—	—	0.6	0.3
5	60	0.7	0.6	0.4	0.4
6	60	0.3	0.2	0.3	0.1

¹ Schultz-Dale responses are expressed as histamine equivalents $\times 10^{-8}$.

Table III. Schultz-Dale responses of guinea pigs exposed by oral ingestion to laundry detergent

Guinea pig No.	Length of exposure days	Responses ¹ alcalase concentration in bath, g/ml			
		10 ⁻⁸	10 ⁻⁷	10 ⁻⁵	10 ⁻³
1	46	—	—	—	—
2	50	0.3	0.3	0.6	0.4
3	52	—	—	—	—
4	58	—	0.1	0.3	0.2
5	64	0.3	0.5	0.2	0.3
6	64	—	—	—	—

¹ Schultz-Dale responses are expressed as histamine equivalents $\times 10^{-8}$.

household use conditions, i.e. inhalation, oral ingestion, intact and abraded skin exposures. Figure 1 shows typical Schultz-Dale responses of the guinea pigs exposed to the detergent. In each case, specific contractions of the jejunum were observed in the presence of alcalase. Note the lack of response in the nonexposed guinea pig jejunum. Tables II–V indicate the Schultz-Dale responses as a function of the length of exposure of the guinea pig to the detergent. It was interesting to observe that, for the most part, the jejunum of these guinea pigs were not sensitized earlier than 50 days of exposure, however marked sensitization occurred

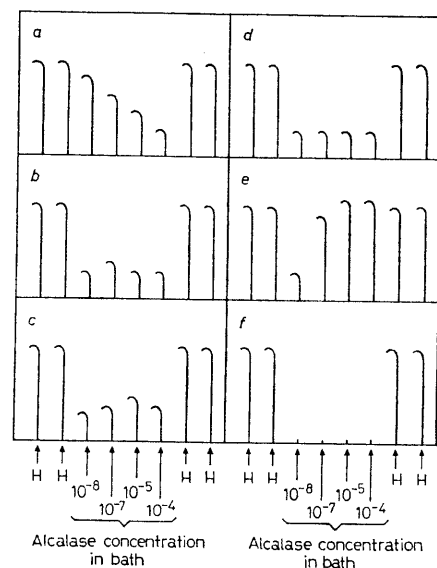


Fig. 1. Schultz-Dale responses of guinea pigs exposed to alkalase containing detergent by: *a* inhalation, *b* oral ingestion, *c* topical and *d* abraded skin application, *e* i.p. injection, *f* unexposed guinea pig. H, histamine.

Table IV. Schultz-Dale responses of guinea pigs exposed by topical application

Guinea pig No.	Length of exposure days	Responses ¹ alkalase concentration in bath, g/ml			
		10 ⁻⁸	10 ⁻⁷	10 ⁻⁶	10 ⁻⁴
1	46	—	—	—	—
2	50	—	—	—	—
3	53	—	—	—	—
4	59	—	—	0.6	0.6

¹ Schultz-Dale responses are expressed as histamine equivalents $\times 10^{-8}$.

Table V. Schultz-Dale responses of guinea pigs exposed by abraded skin application

Guinea pig No.	Length of exposure days	Responses ¹ alkalase concentration in bath, g/ml			
		10 ⁻⁸	10 ⁻⁷	10 ⁻⁶	10 ⁻⁴
1	38	0.2	0.3	—	—
2	39	—	—	—	—
3	53	—	—	—	—
4	59	—	—	—	—
5	65	0.3	0.2	0.2	0.2
6	65	—	—	—	0.4

¹ Schultz-Dale responses are expressed as histamine equivalents $\times 10^{-8}$.

Table VI. RMC responses of sera from guinea pigs exposed to detergent by various routes

Exposure route	Guinea pig No.	Length of exposure days	Cytopathic activity % ¹
Inhalation	1	23	32
	2	23	1
	3	37	35
	4	38	40
Oral ingestion	1	23	15
	2	23	0
	3	45	10
Topical application	1	45	47
Abraded skin application	1	23	3
	2	23	48
	3	37	14
	4	38	12
Non-exposed animals	—	—	5
	—	—	2
	—	—	3

¹ Cytopathic activity represents responses in rat mast cells due to experiments less serum control values. Alkalase was used at a concentration of 0.1 mg/ml.

between 50–60 days. These results indicate that repeated prolonged exposure is a necessary condition for sensitization. Sera from these sensitized animals generally failed to elicit positive 24-hour PCA reactions with alcalase antigen. However, antibodies to alcalase were detected in the sera of these animals using the rat mast cell technique. Table VI summarizes the results obtained with the rat mast cell technique with the sera from the animals exposed by the four routes. It was found that 9 of 12 sera from treated guinea pigs caused morphological changes in rat mast cells in the presence of alcalase. Note the lack of response with sera from 3 control animals. In addition, it was interesting to find that sera from 3 animals on treatment for only 23 days gave positive responses in the rat mast cell test.

Discussion

The results of this study indicate that repeated exposure by various routes of guinea pigs to detergent containing *B. subtilis* enzyme under home usage conditions resulted in sensitization. Although animals exposed by topical and abraded skin application produced antibodies involved in immediate hypersensitivity reactions these same animals failed to produce delayed hypersensitivity reactions. Thus, it is not surprising that GRIFFITH *et al.* [11] failed to detect sensitization in consumers, guinea pigs and rabbits exposed to *B. subtilis* enzymes by topical application since these authors used skin-irritation, an indicator of delayed reactions as the sole criteria of detection. Furthermore, in the present study, during the period to which the guinea pigs were exposed by inhalation route there was no overt evidence of asthma-like episodes. On the other hand, the test animals produced a transient erythema secondary to vasodilation of the ear veins which suggested histamine release. Further studies are required to determine the relationship between allergic respiratory disturbances in these animals, length of inhalation exposure, concentration of airborne enzyme dust and the effect of multiple exposure routes.

Reports have appeared in the literature [6–8] which indicate that some consumers, exposed by various routes to low concentration of enzyme dust in the detergent, can indeed display allergic respiratory manifestation to the enzyme. In order to determine the percent of population showing respiratory symptoms to the enzyme dust, long-term epidemiology studies are required with well-defined allergens relating case histories,

skin-tests, provocation exposure and detection of circulating sensitizing antibodies. Careful consideration must be given to the fact that factory workers have been exposed to high concentration of the enzyme dust for short periods of time. In this case, IgE-type skin-sensitizing antibodies appear to be responsible for the allergic manifestations. Thus a good correlation has been obtained between direct skin-tests and IgE levels in these individuals [15]. Consumers, on the other hand, are chronically exposed to low concentrations of the enzyme dust and by various routes of exposure. It is possible that other types of sensitizing antibodies such as the short, latent binding antibody described by PARISH [16] may play an important role in the enzyme dust sensitivity in the consumer population.

Acknowledgment

The authors wish to thank Mrs. E. MILLARD for her skillful technical assistance and to the Proctor and Gamble Company of Canada, Hamilton, Ontario and United States, Cincinnati, Ohio, for generous gifts of the *B. subtilis* enzymes.

References

- 1 FLINDT, M. L. H.: Preliminary disease due to inhalation of derivatives of *Bacillus subtilis* containing proteolytic enzyme. *Lancet* *i*: 1177 (1969).
- 2 PEPYS, J.; HARGREAVE, F. E.; LONGBOTTOM, J. L., and FAUX, J.: Allergic reaction of the lungs to enzymes of *Bacillus subtilis*. *Lancet* *i*: 1181 (1969).
- 3 NEWHOUSE, M. L.; TAGG, B.; POCOCK, S. J., and MCEWAN, A. C.: Epidemiological study of workers producing enzyme washing powders. *Lancet* *i*: 689 (1970).
- 4 GREENBERG, M.; MILNE, J. F., and WATT, A.: Survey of workers exposed to dusts containing derivatives of *Bacillus subtilis*. *Brit. med. J.* *ii*: 629 (1970).
- 5 HENDRICKS, M. H.: Measurement of enzyme laundry product dust levels and characteristics in human use. *J. amer. Oil Chem. Soc.* *47*: 207 (1970).
- 6 BERNSTEIN, I. L.: Preliminary investigations of sensitization phenomena in populations not exposed to high concentrations of enzyme dust. *J. Allergy* *47*: 97 (1971).
- 7 BELIN, I.; FALSEN, E.; HOBORN, J., and ANDRE, J.: Enzyme sensitization in consumers of enzyme containing washing powders. *Lancet* *ii*: 1153 (1970).
- 8 FALLERONI, A. E. and SCHWARTZ, D. P.: Immediate hypersensitivity to enzyme detergents. *Lancet* *i*: 548 (1971).
- 9 JENSEN, S. E.: Severe dermatitis and 'biological' detergents. *Brit. med. J.* *i*: 299 (1970).

- 10 DUCKSBURY, C. F. J. and DAVE, V. K.: Contact dermatitis in home helps following the use of enzyme detergents. *Brit. med. J. i*: 537 (1970).
- 11 GRIFFITH, J. F.; WEAVER, J. E.; WHITEHOUSE, H. S.; POOLE, R. L.; NEWMANN, E. A., and NIXON, G. A.: Safety evaluation of enzyme detergents. Oral and cutaneous toxicity, irritancy and skin sensitization studies. *Food Cosmet. Toxicol.* 7: 581 (1969).
- 12 PERELMUTTER, L. and KHERA, K.: A study on the detection of human reagins with rat peritoneal mast cells. *Int. Arch. Allergy* 39: 27 (1970).
- 13 PERELMUTTER, L. and LIAKOPOULOU, A.: Detection of IgE mediated immediate hypersensitivity reactions in the sera of ragweed sensitive individuals using rat mast cells. *Int. Arch. Allergy* 40: 481 (1971).
- 14 PERELMUTTER, L. and MILLARD, E.: A modified rat mast cell technique for the detection of human IgE-type reagins. *Int. Arch. Allergy* 40: 675 (1971).
- 15 RITZ, H.: Personal communication (Proctor and Gamble Co., Cincinnati, Ohio).
- 16 PARISH, W. E.: Short-term anaphylactic IgG antibodies in human sera. *Lancet* i: 591 (1970).

Authors' address: Dr. L. PERELMUTTER, Dr. A. LAVALLÉE and Dr. G. S. WIBERG, Immunology Division, Food and Drug Directorate, Tunney's Pasture, Ottawa, Ontario K1A 0L2 (Canada)

Lymphocyte Mitogens of Indigenous Australian Plant Species

C. C. CURTAIN and M. J. SIMONS

CSIRO Division of Animal Health, Animal Health Research Laboratory,
and The Royal Children's Hospital Research Foundation, Parkville, Vic.

Abstract. Saline extracts of seeds from 164 Australian indigenous plant species representing 73 genera and 28 families were tested for their ability to stimulate blast transformation and mitosis in human buffy coat cells. Extracts from 3 species, *Rhagodia crassifolia*, *Bassia decurrens* and *Bauhinia Carronii* were found to be active. The most active extract, from *B. Carronii* was fractionated by isoelectric focussing, by which it was found possible to separate the mitogenic, haemagglutinating and leucoagglutinating activities. The activity of the *B. Carronii* mitogen was not inhibited by antiserum to the phytohaemagglutinin of *Phaseolus vulgaris* (PHA) and its chemical composition and sedimentation co-efficient differed from those published for PHA and the mitogen of the pokeweed (*Phytolacca americana*).

Introduction

The behaviour of peripheral blood small lymphocytes, when cultured in the presence of various stimulating substances, has been the subject of intensive study and discussion in recent years. These substances cause morphological changes characterised by transformation of dense inactive heterochromatin into fibrillar, metabolically active euchromatin [FRENS-TER *et al.*, 1963], the appearance of nucleoli and an increase in basophilia and pyroninophilia of cytoplasm [HIRSCHHORN *et al.*, 1965]. Accompanying these morphological changes are extensive biochemical changes, the most striking of which are increases in the synthesis of protein [BACH and HIRSCHHORN, 1963], in RNA [MCINTYRE and EBAUGH, 1962] and DNA [BENDER and PRESCOTT, 1962]. These events may culminate in mitosis.

Received: September 24, 1971.