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Decrease in susceptibility to oral tolerance induction and occurrence of oral immunization to ovalbumin in 20–38-week-old mice. The effect of interval between oral exposures and rate of antigen intake in the oral immunization

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SUMMARY

Maturation into adulthood, from 8 to 24 weeks of age, significantly influences the induction of oral tolerance in different strains of mice. Animals from strains which are susceptible to the induction of oral tolerance to ovalbumin (OVA) at 8 weeks of age become refractory at 24 weeks of age. Furthermore, in several strains, intermittent exposure to OVA exclusively by gavage resulted in high titres of circulating anti-OVA antibodies. However, the voluntary intake of similar doses of OVA at the same intervals failed to immunize mice of one of the most responsive strains, H-III.

INTRODUCTION

The major route of contact for antigens is evidently the digestive tract. Although this goes almost unrecognized in current medical practice, such daily and massive forms of antigenic exposure must play an important role in the operation of the immune system as a whole.

Antigenic contacts initiated by oral route are thought to induce oral tolerance, a state of relative suppression of specific immune responses to subsequent parenteral injections of the antigen.[1,2] At the same time, they may induce the production of secretory IgA specific antibodies, at the local site of mucosal stimulation as well as at other distant mucosal sites (e.g. ocular, respiratory, genital tract, etc.).[3-5]

Nevertheless, it is unclear whether all the ingested antigenic material induces specific immune tolerance. There is abundant evidence that oral exposure to antigens may result in formation of circulating antibodies. Brandtzaeg[6] and Bartholomeusz[7] reported that high levels of circulating polymeric IgA can be achieved in individuals immunized by the oral route. Oral administration of cholera toxin[8] and syncytial respiratory virus[9] also involve simultaneous appearance of serum IgG and secretory IgA in mucosal sites. In rabbits, ingestion of bovine serum albumin (BSA) results in systemic specific immunization without secretory IgA production.[9] It is still polemic whether systemic antibody production arises from a mucosal or from an extramucosal site.[7]

Abbreviations: H-III, high responder mice of Selection III; i.p., intraperitoneal; OD, optical density; OVA, ovalbumin.

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The possibility of inducing circulating specific antibodies by oral immunization makes it important to distinguish the conditions determining the outcome of the oral administration of antigens.

When orally induced tolerance was studied previously, it was observed that maturation into adulthood, from 8 to 24 weeks of age, significantly affects its induction in C57BL/6J mice.[10] Animals which were susceptible to the induction of oral tolerance of ovalbumin (OVA) at 8 weeks of age became refractory at 24 weeks of age. Moreover, the oral administration of OVA enhanced, instead of suppressing, the specific response after parenteral immunization with the same antigen. Since the protocol for tolerance induction involved parenteral immunization, it was not clear whether the priming effect observed was due to direct oral immunization. The present experiments were performed to expand these observations to other strains of mice as well as to test the immunological consequences of antigenic administration exclusively by oral route.

MATERIALS AND METHODS

Mice

Animals from several inbred strains (A/J, A/Sn, A.BY, BALB/cJ, C3H/HeJ, C3H.SW, CBA) obtained from Dr S. T. Torres (UFF, Niterói, Rio de Janeiro, Brazil) and mice with 'high multispecific reactivity' (Selection III high line, or H-III) obtained from Dr O. A. Sant'Anna (Instituto Biológico, São Paulo, Brazil) were bred in our colonies as well as the hybrid B6D2F_1. The mice were between the ages of 8 and 38 weeks. They were used in groups of four to eight animals, as described in the text.

Antigen

5 × crystallized hen's egg albumin (OVA; Sigma, Kankakee, Il) was used as antigen.

Parenteral immunization

Mice were injected intraperitoneally with 10 μg of OVA mixed with 1 mg $Al(OH)_3$ as an adjuvant in saline. They received a booster injection of 10 μg of OVA in saline 21 days later and were bled 7 days later.

Oral administration

Mice were slightly anaesthetized with ether and given 20 mg of OVA in 0·5 ml of saline by gavage. Control animals received 0·5 ml of saline. Alternatively, mice received 4 mg/ml solution of OVA in water as their exclusive drinking fluid. The average voluntary intake of a mouse is 5 ml in 24 hr;[11] therefore the animals were assumed to receive the same amount of OVA (20 mg) per day.

Antibody assays

Antibody titres to OVA were determined by standard ELISA assay using an automatic ELISA reader (Titertek Multiskan, Finland, 30c). In short, the plates (Hemobag, São Paulo, Brazil) were coated overnight with 2 μg of antigen in 100 μl/well of sodium carbonate buffer, pH 9·6, at 4°. They were then washed with saline containing 0·05% Tween-20 and incubated for 30 min with phosphate-buffered saline (PBS) containing 1% casein and 0·05% Tween-20. They were then washed again, incubated with mouse anti-serum dilutions in PBS, starting at 1/100 for 1 hr, washed again and incubated with peroxidase conjugates of rabbit anti-mouse globulins (IgG+IgA+IgM; Kirkegaard & Perry Labs, Gaithersburg, MD) at 1/1500. Plates were then washed six times, and incubated with H_2O_2 in the presence of orthophenylene diamine (OPD) in sodium citrate buffer, pH 5·0 for 20 min in the dark. The reactions were interrupted by the addition of 20 μl of 10% sodium dodecylsulphate (v/v in water). ELISA scores were computed by running sums of optical densities between 1/100 and 1/10,240 serum dilutions in individual mice. Each number designated ELISA* represents the area delimited by a curve. In Table 1 the curve areas of individual responses are shown; in the other tables and Fig. 1 the mean of a group is presented. Differences in the means of ELISA scores among groups were then determined by two-tailed Student's *t*-tests.

RESULTS

The first experiments were performed using the protocol for inducing oral tolerance as described in previous papers.[1,10,12] Mice were split into two groups. Test groups received 20 mg of OVA in saline by gavage, and control groups only saline as pretreatment on day −7. They were all immunized with OVA intraperitoneally on day 0 and boosted on day 21. All animals were bled 7 days thereafter.

Table 1 shows the effect of maturation into adulthood, on the susceptibility to oral tolerance induction with OVA, on mice of three different strains previously recognized as susceptible: C3H/HeJ, $B6D2F_1$ and A/J. In 7–8-week-old mice of all three strains, oral pretreatment with OVA resulted in significant degrees of tolerance. The magnitude of immune responsiveness of the control (saline pretreated) animals in the three strains was rather similar. When 20–38-week-old mice were used, oral tolerance was still induced in C3H/HeJ and $B6D2F_1$ mice, although less efficiently. However, in older A/J mice, oral tolerance was completely abolished.

Table 1. Influence of age on susceptibility to induction of oral tolerance with OVA. Mice were pretreated by gavage with either saline or OVA 7 days before primary immunization. Total serum anti-OVA antibodies in individual animals were measured by ELISA and the OD curves were integrated. The numbers represent the mean ± SD (n = 5–6) of the integrals

Strains	Age (weeks)	Oral treatment	
		Saline	OVA
C3H/HeJ	8	6480 ± 530	1320 ± 201*
	20	6682 ± 500	4440 ± 728
A/J	7	4632 ± 797	1944 ± 791**
	38	5408 ± 1233	7224 ± 334
$B6D2F_1$	8	5624 ± 210	613 ± 57*
	38	3728 ± 82	1593 ± 108

*$P < 0{\cdot}01$ compared to control (saline); **$P < 0{\cdot}05$ compared to control (saline).

Since a decrease, and even abolition, of susceptibility to oral tolerance induction in older animals was observed, it was decided to test next whether the administration of OVA exclusively by oral route, without any parenteral immunization, would induce serum specific antibodies.

Figure 1 shows that 20–28-week-old mice from several strains, when exposed to OVA exclusively by oral route (gavage) on days −7, 0 and 21, produced circulating anti-OVA antibodies in titres as high as those produced by intraperitoneally

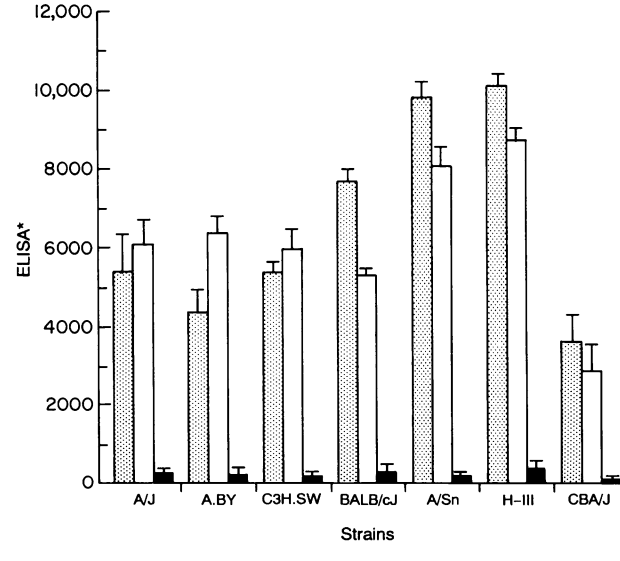

Figure 1. Serum antibodies to OVA induced by oral immunization in 20–28-week-old mice. Mice were treated with OVA either by (▨) i.p. injection on days 0 and 21 or by (□) gavage on days −7, 0 and 21. Untreated mice were used as controls (■). Total serum anti-OVA antibodies in individual animals were measured by ELISA and the OD curves were integrated. The bars represent the mean ± SD (n = 4–7) of the integrals (ELISA*).

Table 2. Influence of age on serum antibody production to OVA after oral immunization. Mice were treated with OVA by gavage on days −7, 0 and 21. Total serum anti-OVA antibodies in individual animals were measured by ELISA and the OD curves were integrated. The numbers represent the integral of each individual curve (ELISA*)

	8 weeks old		24 weeks old	
Strain	Animal	ELISA*	Animal	ELISA*
C3H.SW	1	2371	1	7091
	2	74	2	6878
	3	251	3	6351
	4	147	4	6475
	5	162	5	5871
BALB/cJ	1	10,817	1	6715
	2	1497	2	3975
	3	4408	3	5520
	4	2420	4	5047
	5	145		
	6	1646		
C3H/HeJ	1	5550	1	6480
	2	1363	2	1275
	3	1000	3	764
	4	1090	4	749
	5	675		

Table 3. Effect of different schedules and conditions of antigen administration on serum antibody production to OVA after oral immunization in 20–24-week-old mice. Mice were treated with OVA either by gavage or voluntary ingestion on two different schedules: either on 5 consecutive days (×5 consecutive) or on days −7, 0 and 21 (×3 intermittent). Total serum anti-OVA antibodies in individual animals were measured by ELISA and the OD curves were integrated. The numbers represent the mean ± SD. (n = 4–8) of the integrals

	Schedules and conditions for oral treatment			
	×5 consecutive		×3 intermittent	
Strains	Voluntary ingestion	Gavage	Voluntary ingestion	Gavage
C3H.SW	230 ± 58	140 ± 39*	ND	6932 ± 382
BALB/cJ	131 ± 30	2818 ± 1224	ND	5932 ± 321
H-III	129 ± 15	834 ± 435**	221 ± 13*	4782 ± 124

ND, not done.
*P < 0·001 compared to control (gavage ×3 intermittent); **P < 0·01 compared to control (gavage ×3 intermittent).

immunized mice. None of these strains could be rendered tolerant at this age by oral pretreatment with ovalbumin (data not shown).

The next experiments were designed to investigate which factors were critical for this type of 'oral immunization'.

Table 2 shows the result of an experiment carried out to test whether the age of the animal affected 'oral immunization' in the same way as it affected oral tolerance. Mice of three different strains (C3H.SW, BALB/cJ and C3H/HeJ) were exposed to OVA exclusively by oral route (gavage) on days −7, 0 and 21 when they were either 8 or 24 weeks old. Although C3H/HeJ mice could not be homogeneously immunized by the oral route at either age, in the other two strains (C3H.SW and BALB/cJ), a homogeneously high antibody response at 24 weeks of age and a poor and heterogeneous immune response (only one to three mice out of each group was immunized) at 8 weeks were observed.

The effects were also tested of different schedules and conditions of oral exposure to OVA on the formation of specific antibodies in 20–24-week-old mice. The animals were either exposed to three doses of OVA, on days −7, 0 and 21, and bled on day 28 (designated by ×3 intermittent), or they were exposed to five doses of OVA on 5 consecutive days and bled 7 days thereafter (designated by ×5 consecutive). In both schedules, oral administration was made in two different forms: either by gavage or by voluntary intake. Table 3 shows the responsiveness of three different strains of mice: C3H.SW, H-III and BALB/cJ. In the animals treated by gavage, three intermittent administrations of antigen induced similar antibody responses in all strains. However, five consecutive administrations of the same antigen induced variable responses in the three strains. In C3H.SW mice, there was no detectable antibody formation; in H-III, only one mouse out of five made a moderate response; in BALB/cJ mice, the four animals tested had different patterns of response, ranging from null to a very high level.

Finally, it was investigated whether the voluntary ingestion of OVA, or the intragastric administration of OVA by gavage had similar immunological effects. Mice which voluntarily ingested about 20 mg of OVA/day, during 5 consecutive days, were bled 7 days thereafter. No antibodies were detected in 20-week-old C3H.SW (four mice), H-III (five mice), or BALB/cJ (eight mice in two independent experiments) animals. However, this difference could be better observed when 20-week-old H-III mice were exposed to 20 mg of OVA on days −7, 0 and 21, either by gavage, or by voluntary intake. Whereas administration of OVA by gavage induced significant antibody formation, voluntary ingestion did not. Therefore, the effect of voluntary ingestion of OVA seems to be different from the effect of oral administration of the same antigen by gavage.

DISCUSSION

Oral tolerance can be used as an effective way of preventing undesirable systemic responses in humans, as is the case in pediatric allergy to house dust allergens.[13] There is evidence that repeated oral exposures to the specific antigen may interfere even with the ongoing immune response.[11,14] The development of clinical trials of this kind is hindered by the lack of precise knowledge of the factors that may eventually heighten, instead of suppressing, specific immune responsiveness after oral contact with an antigen.

Our data clearly show that either oral tolerance or the production of circulating anti-OVA antibodies may result from oral exposures to OVA. The factors that determine which of

these outcomes will prevail include age, mouse strain, interval between oral exposures, and the rate of antigen intake.

Among those factors affecting oral tolerance, age is certainly the most described. The decrease in susceptibility to tolerance with increasing age has been reported on New Zealand Brown (NZB), BALB/c and C57BL/6 mice.[15,16] In those experiments, however, the animals were senile, over 1 year old, when tested. It was previously observed that not only senescence but maturation into adulthood (from 8 to 24 weeks of age) is a limiting factor for tolerance induction by oral route in C57BL/6J mice.[10] There is also evidence that both the B-cell population[17] and the T-cell population[18] in 24–28-week-old mice are more refractory to tolerance induction than in 6–8-week-old mice. The 40–44-week-old animals are even more resistant.

The present results confirm this observation and suggest that there is a particular period in the mouse lifetime when oral tolerance is prone to occur. That period seems to be after immunological maturation[12] but before full adulthood.

All three strains tested presented a high susceptibility to oral tolerance induction at 8 weeks of age whereas at 24 weeks tolerance was only partial in $B6D2F_1$ and C3H/HeJ mice and completely abolished in A/J mice. The decrease in the susceptibility to tolerance induction observed in the present experiments and in a previous study using C57BL/6J mice,[10] could be parallel to an increased susceptibility to immunization in adult mice exposed to antigen by the oral route. To test this possibility, mice were treated with antigen exclusively by oral administration.

Figure 1 shows that 20–24-week-old mice after three exposures to OVA, exclusively by gavage, display high levels of anti-OVA antibodies in serum. In seven mouse strains tested, the levels of antibody production were comparable to those induced by parenteral immunization.

As shown in Table 2, intermittent oral exposure to OVA in mice of two different ages (8 and 24 weeks) and three different strains induced antibodies regularly in the older mice in two out of the three strains (C3H.SW and BALB/cJ). C3H/HeJ could not be consistently immunized by oral route in either 8 or 24 weeks of age. Although the results do not show the drastic effect of age as in the oral tolerance experiments (Table 1), they suggest that the age of the animals is involved in the homogeneous high specific response observed in Fig. 1 and Table 2.

The results of current studies are consistent with the reported data that suggest there is a decrease in the susceptibility to oral tolerance induction in 24–44-week-old mice and a parallel increase in the susceptibilty to induction of serum-specific antibodies after oral treatment with antigen.[15–19]

Further studies are still underway in order to investigate the isotype and the origin of the serum antibodies observed after oral immunization in our protocol. Recent data by Bartholomeusz *et al.*[7] suggest that a serum polymeric IgA response can be induced by a typhoid vaccine delivered either orally or parenterally and that the pIgA in serum are not derived from the intestinal mucosa but from extra-mucosal sites.

Age is not the only factor that affects the outcome of oral administration of antigens. The interval between oral treatments and the rate of antigen intake are clearly decisive for this outcome.

In Table 3 it is shown that 24-week-old mice of three different strains (C3H.SW, BALB/cJ and H-III) individually display very heterogeneous responses (BALB/cJ mice), or completely failed to respond (C3H.SW and H-III mice) when exposed to 20 mg of OVA by gavage during 5 consecutive days. Therefore, elimination of the intervals between exposures prevents the oral immunization, or makes it a rare event.

Since some of the BALB/cJ mice could be immunized in the 5 consecutive-day schedule, although in a very uneven pattern, it was decided to test whether the rate of antigen intake might have been contributing to the oral immunization. So far, OVA had been administered exclusively by gavage. However, in natural conditions, especially in rodents, dietary compounds are ingested continuously and in small amounts during the day. It was observed that if mice of three different strains (C3H.SW, BALB/cJ and H-III) were treated during 5 consecutive days with the same amount of OVA (20 mg/24 hr) by voluntary ingestion, they showed no circulating anti-OVA antibodies.

A more significant difference was observed when 24-week-old H-III mice were given OVA either by gavage or by voluntary ingestion in three intermittent administrations (Table 3). Whereas gavage stimulated antibody responses, voluntary ingestion induced no detectable response. This strongly suggests that the schedule and the conditions of oral antigen administration to achieve 'oral immunization' are opposite to those inducing oral tolerance; they are, actually, similar to those required for parenteral immunization. Oral immunization seems to require an intermittent and fast antigen intake, whereas oral tolerance requires a gradual and continuous administration of the antigen.[20]

The most efficient way to induce oral tolerance is the continuous administration of the antigen in small doses. Experiments in mice[11] and guinea-pigs[21] demonstrated that ingestion of OVA for several consecutive days was able to suppress the delayed hypersensitivity and the serum antibody response to a subsequent injection of the antigen in complete Freund's adjuvant. The animals were anaphylatically sensitized if the antigen was introduced in their diet all at once.

According to Stokes *et al.*,[20] tolerance induction is related to a gradual and continuous absorption of the antigen. They showed in CBA and SWR/J mice that administration of 25 mg of OVA by intragastric route for 14 days is unable to induce tolerance but if the antigen was voluntarily ingested throughout the day at the same amounts, the animals could be rendered tolerant. The continuous contact with the antigen would then be necessary to trigger the tolerance circuits. On the other hand, Pomeranz & Normal[22] described opposite requirements for oral immunization. They observed that, although the digestive tract was not an efficient route to induce serum antibody production, this difficulty could be overcome by altering the rates of antigen absorption. Using a solvent which promotes a rapid absorption along with a large dose of antigen and fasting animals, they could induce anaphylactic reactions by oral route as efficiently as by intravenous injections. In these experiments an attempt was made to avoid manipulations such as chemical solvents and fasting conditions to show that the simple change in the intervals and in the rate of antigen administration can lead to a high serum antibody response.

Many studies of oral administrations of antigen are performed with subsequent parenteral test immunizations. This may mask observations of the effects of oral exposures to antigen, in themselves. It is shown here that antigen exposures exclusively by oral route can lead to serum antibody levels comparable to those achieved by parenteral immunizations with

$Al(OH)_3$ adjuvant. It was concluded that oral immunization occurs optimally at a certain age interval, requires special conditions of antigenic administration, and seems to be a very rare event in the natural physiological contacts of the immune system with antigens.

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REFERENCES

1. Vaz N.M., Maia L.C.S., Hanson D.G. & Lynch J.M. (1977) Inhibition of homocytotropic antibody responses in adult mice by previous feeding with the specific antigen. *J. Allergy clin. Immunol.* **60,** 110.
2. Miller S.D. & Hanson D.G. (1979) Inhibition of specific immune responses by feeding protein antigens. IV. Evidence for tolerance and specific active suppression of cell-mediated immune responses to ovalbumin. *J. Immunol.* **123,** 2344.
3. Challacombe S.J. & Tomasi T.B. (1980) Systemic tolerance and secretory immunity after oral immunization. *J. exp. Med.* **152,** 1459.
4. Phillips-Quagliata J.M. & Lamm M.E. (1988) Migration of lymphocytes in the mucosal immune system. In: *Migration and Homing of Lymphoid Cells* (ed. A. J. Husband), Vol. 2, p. 53. CRC Press Inc., Boca Raton, FL.
5. McDermont M. & Bienenstock J. (1979) Evidence for a common mucosal immunological system. I. Migration of B immunoblasts into intestinal, respiratory and genital tissues. *J. Immunol.* **122,** 1892.
6. Brandtzaeg P. (1976) Studies on J chain and binding site for secretory component in circulating human B cells. II. The cytoplasm. *Clin. exp. Immunol.* **25,** 59.
7. Bartholomeusz R.C.A., Forrest B.D., Labrooy J.T., Ey P.L., Pyle D., Shearmabn D.J.C. & Rowley D. (1990) The serum polymeric IgA antibody response to typhoid vaccination; its relationship to the intestinal IgA response. *Immunology,* **69,** 190.
8. Elson C.D. & Ealding W. (1984) Systemic and mucosal immunity in mice after mucosal stimulation with cholera toxin. *J. Immunol.* **132,** 2736.
9. Peri B.A., Theodore C.M., Losonsky G.A., Fishaut J.M., Rothberg R.M. & Ogra P.L. (1982) Antibody content of rabbit milk and serum following inhalation or ingestion of respiratory syncytial virus and bovine serum albumin. *Clin. exp. Immunol.* **48,** 91.
10. Rios M.J.C., Pereira M.A.C., Lopes L.M., Faria A.M.C., Gontijo C.M., Castanheira E.B. & Vaz N.M. (1988) Tolerance induction and immunological priming initiated by mucosal contacts with protein antigens in inbred strains of mice. *Braz. J. med. Biol. Res.* **21,** 825.
11. Saklayen M.G., Pesce A.J., Pollak V.E. & Michael J.G. (1984) Kinetics of oral tolerance: study of variables affecting tolerance induced by oral administration of antigen. *Int. Arch. Allergy appl. Immunol.* **73,** 5.
12. Hanson D.G. (1981) Ontogeny of orally-induced tolerance to soluble proteins in mice. I. Priming and tolerance in newborns. *J. Immunol.* **127,** 1518.
13. Challacombe S.J. & Tomasi T.B. (1987) Oral tolerance. In: *Food Allergy and Intolerance* (eds J. Brostoff and S. J. Challacombe), p. 255. Baillière Tindall, London.
14. Bloch K.J., Perry R., Bloch M. & Walker W.A. (1983) Induction of partial systemic tolerance in primed rats subjected to prolonged oral administration of antigen. *Ann. N.Y. Acad. Sci.* **409,** 787.
15. Staples P.J. & Talal N. (1969) Relative inability to induce tolerance in adult NZB and (NZB × NZW) F1 mice. *J. exp. Med.* **130,** 123.
16. Habicht G.S. (1980) Tolerance to human IgG in aged C57BL/6 and Balb/c mice. *Federation Proc.* **39,** 2118.
17. Doken J., Weksler M.E. & Siskind G.W. (1980) Effect of age on ease of B-cell tolerance induction. *Cell. Immunol.* **55,** 66.
18. Dekkruyff R.H., Kan E.A.R., Weskler M.E. & Siskind G.W. (1980) Effect of aging on the T cell tolerance induction. *Cell. Immunol.* **56,** 58.
19. Kawanishi H. & Kiely J. (1987) Immunoregulatory defects in murine aged Peyer's patches. *Eur. J. Immunol.* **17,** 1223.
20. Stokes C.R., Swarbrick E.T. & Soothill J.F. (1982) Genetic differences in immune exclusion and partial tolerance to ingested antigens. *Clin. exp. Immunol.* **53,** 678.
21. Heppel L.M.J. & Kilshaw P.J. (1982) Immune responses of guinea pigs to dietary protein. I. Induction of tolerance by feeding with ovalbumin. *Int. Arch. Allergy appl. Immunol.* **68,** 54.
22. Pomeranz J.R. & Norman P.S. (1966) Concomitant anaphylactic sensitization and contact unresponsiveness following the infusion of feeding of pycryl chloride to guinea pigs. *J. exp. Med.* **124,** 69.