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Irradiated mice lose the capacity to 'process' fed antigen for systemic tolerance of delayed-type hypersensitivity

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SUMMARY

'Intestinal antigen processing' is a function of the gastro-intestinal tract whereby shortly after an animal has been fed an immunogenic protein antigen, such as ovabumin (OVA), a tolerogenic form of the protein is generated and can be detected in the circulation. The effect of damage to the intestinal epithelium on the processing of OVA has been examined in lethally irradiated mice. Irradiated animals were fed 25 mg OVA and their serum collected 1 h later. When this serum was transferred intraperitoneally into naive recipient mice, this did not induce the typical suppression of systemic delayed-type hypersensitivity. Results were similar when the serum donors were at 2 days after irradiation, with crypt hypoplasia, and at 5 days after irradiation when there was reactive crypt hyperplasia. However reconstitution of donors with normal spleen cells immediately after irradiation restored their capacity to generate a tolerogenic form of the antigen. Immunoreactive OVA was detected by ELISA in both tolerizing and non-tolerizing sera, and the immunological properties of these sera were not related to serum levels of OVA after feeding. Thus subtle immunochemical alterations in the nature of a protein antigen are likely to be more important than the quantity of absorbed antigen, in influencing systemic cell-mediated immune responses after feeding. The lack of generation of a tolerogenic form of the protein in irradiated mice, unrelated to the pattern of epithelial cell kinetics, and the restoration of this function by normal spleen cells, suggests that lymphoid cells may be involved in the phenomenon of antigen processing.

Keywords oral tolerance irradiation intestinal epithelium gut processing

INTRODUCTION

The phenomenon of oral tolerance affects both humoral and cell-mediated immunity (CMI). For example, mice fed the soluble protein antigen ovalbumin (OVA) before systemic immunization with that antigen, show suppression of serum antibody levels and also of delayed-type hypersensitivity (DTH) (Miller & Hanson, 1979; Mowat *et al.*, 1982). The suppression of systemic CMI is via cyclophosphamide-sensitive suppressor cells activated by a tolerogenic form of OVA which is present in the systemic circulation 1 h after feeding (Strobel *et al.*, 1983; Bruce & Ferguson, 1986a,b). The OVA which is fed to mice is immunogenic, but is altered as it crosses the intestinal mucosa and enters the systemic circulation. 'Gut-processed' OVA in the serum of adult mice

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induces systemic tolerance of CMI, but not of serum antibody responses, in naive animals (Bruce & Ferguson, 1986a,b).

It is widely recognized that the high permeability of immature or diseased intestine may allow ingress of larger than normal amounts of antigen after feeding, but the possible influences of disease processes on the immunoregulatory properties of absorbed antigen have not previously been considered. We have therefore examined the capacity of damaged intestine to process the protein antigen OVA, to render it tolerogenic for systemic DTH responses in mice.

Whole-body irradiation at a lethal dose of 10 Gy was chosen as a means of inflicting gut damage. The effects were assessed at 2 and 5 days after irradiation by analysis of body weight, intestinal histology and morphometry of villi and crypts. The quantity of antigen absorbed from the gut was determined by measuring immunoreactive OVA in the serum after feeding. The effect of radiation injury on intestinal antigen processing was examined in serum transfer experiments where sera obtained from irradiated OVA-fed donors and from various groups of controls were injected into naive recipients. These animals were subsequently immunized and tested *in vivo* for DTH to OVA. This allowed us to establish whether the passive transfer of absorbed OVA had rendered the mice tolerant.

Some types of lymphocytes are exquisitely sensitive to radiation (Anderson & Warner, 1962). Although it seemed likely that the gut epithelium was the critical component in antigen processing, a possible contribution by radiation-sensitive lymphocytes in the gut was examined by using irradiated spleen cell-reconstituted mice.

MATERIALS AND METHODS

Animals

Female BDF_1 mice (crosses between C57BL/6J females and DBA/2 males), 6–8 weeks old, were used for all experiments. These were maintained on a standard laboratory diet (CRM(X); Labsure Ltd) which contains no chicken or egg proteins. There were five or six animals in each experimental group, apart from the serum donors in which each group had 20 mice.

Antigens

Ovalbumin (OVA), (chicken egg albumin, Grave V; Sigma Chemical Co) was used throughout the experiments.

Assessment of systemic immunity

Immunization. Mice were immunized with 100 μg OVA emulsified in 0·05 ml complete Freund's adjuvant (CFA) (H37Ra; Difco) injected into the right hind footpad.

Cell-mediated immunity. This was measured 21 days after immunization by eliciting a DTH reaction in the left hind footpad. Mice were injected with 100 μg OVA dissolved in 0·05 ml saline, and the increment in footpad thickness was measured 24 h later with skinfold thickness calipers (Pocotest-A; Carobronze Ltd).

Irradiation of mice

Mice were placed inside a perspex jig, and were irradiated at a rate of 0·38 Gy/min to give a total dose of 10 Gy from a 250 megavolt orthovoltage X-ray source (Siemens Orthovolt). Cucumber was added to their diet to improve hydration and they were housed in clean cages with autoclaved shavings.

Spleen cell reconstitution

A sterile suspension of spleen cells from female BDF_1 mice was prepared in RPMI 1640 medium (Flow Laboratories Ltd). Trypan Blue dye exclusion was used to identify viable cells. The concentration was adjusted to 5×10^8 viable cells per ml and recipient animals were injected i.v. with 0·05 ml (25×10^6 cells) of the suspension.

Serum transfer procedure

Donor mice were fed by intragastric intubation with either 25 mg OVA dissolved in 0·2 ml 0·15 M NaCl or saline only. At 60 ± 5 min after feeding, the mice were bled out from the axillary vein and artery. The serum obtained was pooled according to experimental groups and 0·8 ml of serum was injected i.p. into each of six recipients. Seven days later, recipient mice were immunized with 100 μg OVA in CFA and after a further 3 weeks they were assessed for systemic cell-mediated immunity. Mice which displayed responses significantly lower ($P < 0·05$) than those observed in OVA-immunized control mice are referred to as being tolerant.

Serum transfer experiments

Groups of BDF_1 mice were either left untreated or given 10 Gy irradiation. At 2 or 5 days after irradiation, the mice were weighed, given a single intragastric feed of either 25 mg OVA or 0·2 ml saline and were bled out 1 h later. A sample of serum was reserved for assessment of OVA by ELISA. Samples of jejunum were collected from some of the serum donors, and processed for histology and microdissection as described below. The sera were pooled according to experimental groups and injected into recipients. These were immunized with OVA 1 week later and were assessed for systemic DTH to OVA 3 weeks after immunization, as described above. In experiments where mice were to be reconstituted with spleen cells, an injection of 25×10^6 cells was given i.v. within 3 h after irradiation.

Detection of OVA in mouse serum

An ELISA technique (Bruce & Ferguson, 1986b) was used to detect and measure immunoreactive OVA in mouse serum.

Histology

Samples of jejunum were taken at 10 cm from the pylorus, fixed in 10% buffered formalin, embedded in wax, and 5 μm sections were cut and stained with haematoxylin and eosin. IEL counts were performed at $\times 1000$ magnification and expressed as IEL per 100 villus epithelial cells (Ferguson & Murray, 1971).

Microdissection and measurements of villi and crypts

Pieces of jejunum were fixed in Clarke's fixative and transferred to 75% ethanol for storage. Specimens were stained in bulk by the modified Feulgen reaction and examined in a dissecting microscope. A single villus and associated crypts were cut out with a cataract knife, placed on a slide in a drop of 45% acetic acid, covered with a coverslip and measured by using a previously calibrated eyepiece micrometer (Ferguson *et al.*, 1977). In each specimen, the lengths of 10–15 villi and crypts were measured.

RESULTS

Intestinal injury induced by irradiation. When examined at both 2 and 5 days after irradiation, mice had lost weight and appeared dehydrated. At 5 days some animals had diarrhoea. Measurements of the jejunal mucosa showed hypoplasia, with short villi and crypts at 2 days, whereas at 5 days, villi were of normal length and crypts were longer than normal (Table 1). Formal counts of mitotic figures and crypt cell production rate were not done but abundant mitotic figures were noted in the crypts at 5 days, indicating that regeneration of the gut epithelium was in progress.

There was evidence of severe intestinal lymphoid depletion in irradiated mice. At post mortem, the Peyer's patches were smaller than normal at 2 days after irradiation and were invisible by 5 days. Counts of IEL were significantly lower than the control in both irradiated groups (Table 1).

Intestinal processing of OVA is abrogated by irradiation. Irradiated mice were used as donors in a serum transfer experiment as outlined in Table 2. The DTH responses in recipients of serum from saline or OVA-fed donors which had been irradiated 2 or 5 days before feeding or were

Table 1. Effect of irradiation on lengths of villi and crypts and on counts of jejunal intraepithelial lymphocytes in BDF_1 mice (mean ± s.d. before, and at 2 and 5 days after 10 Gy irradiation)

	Untreated ($n=5$)	2 Days after irradiation ($n=6$)	5 Days after irradiation ($n=5$)
Villus length (μm)	576±41	435±39*	536±56
Crypt length (μm)	156·8±7·1	94·2±8·0*	323±28·2*
IEL/100 epithelial cells	11·3±0·9	7·0±0·8*	4·4±0·9*

* $P<0.01$ when compared with unirradiated controls.

Table 2. Protocol for serum transfer experiment with irradiated serum donors

Serum donors (day)			Serum recipients (day)		
−5	−2	0	0	7	21
—	—	serum i.g. bled at 1 h	serum i.p.	OVA/CFA immunized	DTH skin test
—	—	OVA i.g. bled at 1 h	serum i.p.	OVA/CFA immunized	DTH skin test
—	10 Gy	saline i.g. bled at 1 h	serum i.p.	OVA/CFA imunized	DTH skin test
—	10 Gy	OVA i.g. bled at 1 h	serum i.p.	OVA/CFA immunized	DTH skin test
10 Gy	—	saline i.g. bled at 1 h	serum i.p.	OVA/CFA immunized	DTH skin test
10 Gy	—	OVA i.g. bled at 1 h	serum i.p.	OVA/CFA immunized	DTH skin test

unirradiated, are summarized in Fig. 1. Transfer of serum from non-irradiated OVA-fed mice suppressed the DTH response in recipients, but serum obtained from donors which had been fed OVA 2 or 5 days after irradiation failed to tolerize for DTH.

Restoration of intestinal antigen processing with normal spleen cells. The failure to generate a tolerogenic form of OVA after irradiation was associated with intestinal mucosal injury and severe lymphoid depletion in irradiated mice. The relevance of the lymphoid component of irradiation damage was examined by reconstituting irradiated serum donors with 25×10^6 normal BDF_1 spleen cells shortly after irradiation.

Reconstituted irradiated mice lost weight after irradiation but they appeared generally healthier than unreconstituted animals. Specimens for histological examination were taken from two untreated, two irradiated and two irradiated, spleen cell-reconstituted mice. The effects of irradiation on general morphology were similar with and without reconstitution, but IEL counts were low in the two irradiated mice and normal in those which had been reconstituted (Table 3).

Reconstituted irradiated mice were fed saline or OVA and their serum transferred into recipients whose DTH responses are summarized in Fig. 2. As before, DTH was suppressed in recipients of serum from non-irradiated, OVA-fed donors. A novel finding was suppression of DTH in recipients of serum from both groups of OVA-fed mice which had been irradiated and reconstituted with spleen cells. Spleen cell reconstitution therefore restored the ability of irradiated, OVA-fed mice to transfer tolerance for DTH with serum.

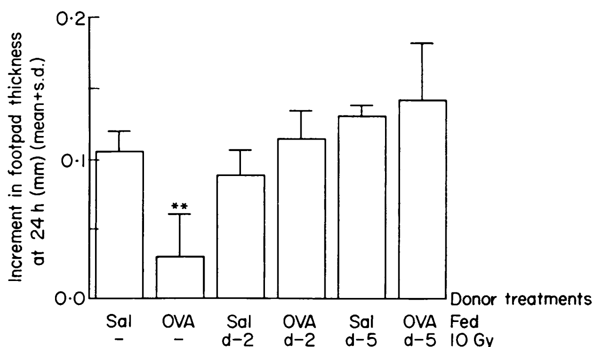

Fig. 1. Systemic DTH responses of recipients of serum from saline or OVA fed donors: effects of irradiation of donors with 10 Gy X-ray. Values shown represent the mean 24 h increment in footpad thickness (mm; mean + s. d.) after OVA challenge in serum recipients at 3 weeks after systemic immunization. All serum donors fed on day 0; d-2, donors irradiated 2 days before feeding; d-5, donors irradiated 5 days before feeding. $^{**}P<0{\cdot}01$.

Table 3. Effects of irradiation and reconstitution with 25×10^6 spleen cells on counts of jejunal intraepithelial lymphocytes in BDF_1 mice (results from two mice in each group)

	Untreated	2 days after irradiation	2 days after irradiation + reconstitution
IEL/100 epithelial cells ($n=2$)	12·1, 11·5	3·95, 5·0	11·3, 11·1

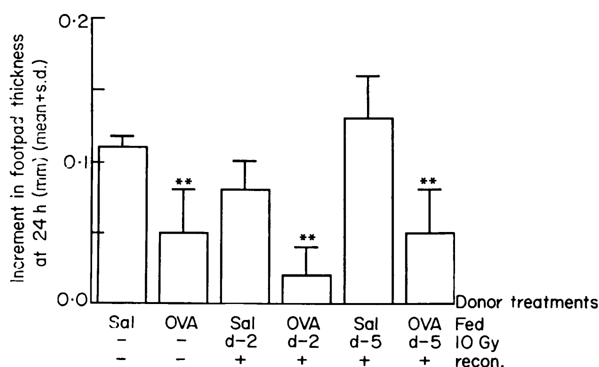

Fig. 2. Systemic DTH responses of recipients of serum from saline or OVA fed donors: effects of irradiation (10 Gy) and spleen cell reconstitution of donors. Values shown represent the mean 24 h increment in footpad thickness (mm; mean + s.d.) following antigen challenge. All serum donors fed on d.0; day-2, donors irradiated 2 days before feeding; d-5, donors irradiated 5 days before feeding; recon, reconstituted donors. $^{**}P<0{\cdot}01$.

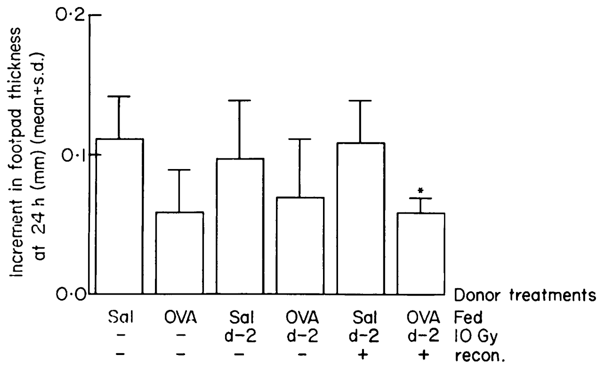

Fig. 3. Systemic DTH responses of recipients of serum from saline or OVA fed donors: effects of irradiation (10 Gy) with and without spleen cell reconstitution of donors. Values shown represent the mean 24 h increment in footpad thickness (mm; mean + s.d. after antigen challenge. All serum donors fed on day 0; d-2, donors irradiated 2 days before feeding; d-5, donors irradiated 5 days before feeding; recon, reconstituted donors. ‡$P < 0·05$.

In order to ensure that the effect of spleen cell reconstitution was reproducible, a further serum transfer experiment was performed at a single time point, 2 days after irradiation. Serum donor mice were either untreated or exposed to 10 Gy irradiation, after which half of the irradiated animals were reconstituted with spleen cells as described above. Two days later these donor mice were fed either saline or OVA, bled out and the serum transferred into naive recipients which were immunized and tested for DTH. Results are shown in Fig. 3. In this experiment, the positive control, suppression of DTH by serum transferred from OVA-fed, unirradiated mice, did not reach statistical significance, but results in the irradiated animals confirmed the observations described above, with no tolerance in mice receiving serum from OVA-fed, irradiated donors, and transfer of tolerance for DTH by serum from OVA-fed animals which had been irradiated and spleen cell reconstituted.

Immunoreactive OVA in tolerizing and non-tolerizing serum. We have previously demonstrated that the presence of OVA, detected by an ELISA in the serum of mice 1 h after a single feed, is associated with the ability of that serum to induce tolerance in recipients (Bruce & Ferguson, 1986b). Immunoreactive OVA was measured by ELISA in the serum of irradiated mice (serum which did not transfer tolerance) and of irradiated, spleen cell-reconstituted mice that were able to generate a serum tolerogen. Only one post-irradiation time point (2 days) was examined in the reconstituted animals.

The results in Table 4 show that OVA was present in the serum of irradiated mice 1 h after feeding and therefore the loss of tolerogenicity associated with this serum was not the result of a lack of antigen. The presence or absence of its tolerogenic effects did not correlate with the amount of immunoreactive OVA in the serum.

DISCUSSION

Irradiation damage to the small intestinal mucosa of the mice used for these experiments was confirmed by microdissection and histological examination. The findings were in keeping with published reports of crypt hypoplasia shortly after irradiation, followed by a regenerative wave of crypt hyperplasia (Quastler & Hampton, 1962). Animals which were compromized by recent irradiation failed to generate a serum tolerogen after OVA feeding and this capacity was restored by spleen cell reconstitution.

We have demonstrated previously that tolerogenic gut-processed OVA in mouse serum can be detected by ELISA (Bruce & Ferguson, 1986b) and have shown indirectly that the induction of tolerance of CMI in serum transfer experiments is not related to the quantity of systemically

Table 4. OVA (by ELISA) in serum of BDF_1 mice at 1 h after oral administration of 25 mg OVA; OVA detected before, and at 2 days and 5 days after 10 Gy X-ray; effect of spleen cell reconstitution shown at 2 days after irradiation (results are from two experiments)

	Serum immunoreactive OVA (ng/ml)			
	Untreated	2 days after irradiation	2 days after irradiation (reconstituted)	5 days after irradiation
Experiment I				
mean ± s.e. ($n = 10$)	63·7 ± 50·2	228 ± 162	—	235 ± 140
Experiment II				
pooled sera ($n = 20$)	158·4	56·2	35·5	—

available antigen (Strobel *et al.*, 1983; Bruce & Ferguson, 1986a). The experiments described in this report strengthen the concept that suppression of DTH by gut-processed OVA is not a function of the quantity of immunoreactive OVA but rather that gut-processing involves some physico-chemical alteration to the antigen. We have shown previously that tolerogen is removed from the serum of healthy, OVA-fed mice by absorption of serum with anti-OVA IgG bound to Sepharose (Bruce & Ferguson, 1986b). The loss of tolerogenicity in serum of irradiated, OVA-fed mice without loss of immunoreactive OVA shows that, although binding epitopes for anti-OVA antibodies are present on the tolerogenic form of OVA, they are not directly associated with its immunosuppressive properties.

Experiments by other workers, on the immune response of low-responder strains of mice to hen egg lysozyme (HEL), have shown that the dominant T cell-mediated suppression induced by HEL is due to the presence of a suppressor determinant associated with an amino-terminal peptide (Wicker *et al.*, 1984). If suppression of CMI following the oral administration of protein antigens is indeed due to a subtle change in the molecule, one method of achieving this would be if intestinal processing of OVA involved the exposure of a suppressor determinant without other major structural alteration.

The normal intestinal processing of OVA was abrogated by 10 Gy whole-body irradiation and this was observed during crypt hypoplasia at 2 days after irradiation and also during crypt hyperplasia at 5 days. It is therefore unlikely that loss of intestinal processing was associated with a particular change in the enterocytes or in epithelial cell kinetics. We considered the possibility that damage to the pancreas or to enterocyte brush border enzymes might have influenced the properties of OVA absorbed into the circulation, by virtue of impaired luminal proteolysis or peptidase deficiency. However, this seems unlikely, for OVA administered to mice via the colon as an enema, and therefore removed from the effects of luminal gastric and pancreatic enzymes, induced systemic tolerance (Strobel, 1983).

In the experiments described above, responses of serum recipients *in vivo* measure the overall status of the animal—tolerant, primed, or, occasionally (as in mice aged 1–7 days (Strobel & Ferguson, 1984)) no net effect. The immune status is affected by various helper and suppressor stimuli, antigen-specific and non-specific, cellular and via mediators. It is conceivable that under normal circumstances both immunogenic and tolerogenic forms of OVA enter the bloodstream, the predominant form being tolerogenic, and that irradiation damage to the intestine allows ingress of a higher proportion of the immunogenic material, such that the effects of co-existing tolerogen are overridden. This would be similar to parenteral injection of native OVA into normal mice at the time of OVA feeding, a regime which prevents tolerance induction (Hanson *et al.*, 1979). However, our observations that serum tolerogen in OVA-fed mice is of a similar molecular weight to native OVA, with neither priming nor tolerance transferred by high or low molecular weight fractions of serum (Bruce & Ferguson, 1986a), argue against an explanation based on molecular heterogeneity.

The capacity of the gut of irradiated mice to render OVA tolerogenic was restored by an injection of spleen cells given within a few hours of irradiation. Continued weight loss of irradiated, reconstituted mice indicated that the animals were not spared the enteropathic effects of radiation despite a degree of re-population of the gut-associated lymphoid tissue. The restoration of gut processing could be via lymphoid or accessory cells interacting with antigen in association with class II major histocompatibility antigens on the surfaces of enterocytes (Ferguson, 1987).

Inappropriate regulation of mucosal and systemic immune responses to enteric antigen may evoke potentially harmful immune reactions, such as the enteropathy of mucosal DTH (Ferguson, 1987). The experiments described in this report highlight the close associations between intestinal physiology, digestive function and immunoregulation, and may have clinical parallels, not only in patients with damage due to irradiation, but also in intestinal infections and after cancer chemotherapy.

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