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The early appearance of specific cytotoxic T cells in murine gut mucosa

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SUMMARY

Using a new technique for isolating lymphoid cells from the lamina propria of murine small intestine, we have examined the appearance of specific cytotoxic T cells in the gut following intraperitoneal immunization with an allogeneic tumour. Specific cytotoxic T cells appeared in the lamina propria at a time when there are very few cytotoxic lymphocytes in any of the organized lymphoid tissues. Greater levels of cytotoxicity were found in the gut compared with any other site for at least 3 weeks following a single injection of tumour cells.

INTRODUCTION

In animal systems, most studies of specific cytotoxic cell reactions have concentrated on either organized lymphoid tissue like the spleen or lymph nodes or peritoneal exudates (Brunner *et al.*, 1970; Jones & Penfold, 1979; Sanderson, 1976). In humans, technical considerations have limited most studies to the natural killer (NK) or antibody-dependent cellular cytotoxic (ADCC or K cell) activities of peripheral blood lymphocytes. Recently, however, it has been reported that lymphoid cells extracted from human ileal or colonic mucosa exhibit low K cell activities compared with peripheral blood lymphocytes (Clancy & Pucci, 1978; Bland *et al.*, 1979; Bookman & Bull, 1979). Further, K and NK cell activities of guinea-pig lymphocytes extracted from the epithelial layer of the small intestine (intraepithelial lymphocytes) are greater than those of lymphocytes extracted from the lamina propria. This trend was also found in assays of mitogen-induced cellular cytotoxicity in the presence of phytohaemagglutinin, a non-specific process known to be mediated by T cells (Arnaud-Battandier *et al.*, 1978). Experimentally, it is of great interest to examine cytotoxic cell responses in the small intestines of animals bearing tumour loads, particularly since neoplasms of the small bowel are very rare (Southam, 1974). We are unaware of any previous observations on specific cytotoxic T cell responses in the gut but here we report the interesting fact that cytotoxic T cells appear in the lamina propria of mouse small intestine very soon after the intraperitoneal inoculation of tumour cells.

MATERIALS AND METHODS

Animals. C57Bl 10 ScSn (H-2^b), CBA/ca (H-2^k) and DBA/2 (H-2^d) mice, 8–12 weeks old, were obtained from department stocks. Within any one experiment, age- and sex-matched animals were used.

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Tumour cells. P-815 mastocytoma cells, originally derived from DBA/2 mice, were kindly supplied by Dr Colin Sanderson, CRC, Northwick Park, Harrow. EL-4 lymphoma cells from C57Bl mice and L-1210 lymphoma cells from DBA/2 mice were supplied by the Department of Biochemistry, Glasgow University. TLX-5 lymphoma cells from CBA mice were supplied by the Department of Surgery, Western Infirmary. Cells were maintained by weekly ascites passage of 3×10^7 cells in syngeneic animals. YAC-1, a subline of YAC lymphoma from A strain mice, was supplied by Flow Laboratories and grown *in vitro*. For cytotoxicity experiments, cells were maintained in suspension culture in RPMI 1640 with 10% new-born calf serum (NCS; GIBCO BIOCULT Ltd). Cells were always subcultured 24 hr before use to ensure that they were in a logarithmic growth phase.

Inoculations. C57Bl mice were given 3×10^7 P-815 cells intraperitoneally in 0·5 ml of phosphate-buffered saline (PBS). Control animals received PBS only.

Cell suspensions. At certain times after inoculation of tumour cells, cell suspensions were prepared from various organs. The spleen and Peyer's patches were processed by forcing fragments through a stainless-steel sieve. Bone marrow suspensions were prepared by flushing out femurs with medium. Mesenteric lymph node (MLN) cells were prepared by teasing the nodes apart in medium with a sterile scalpel blade. All cells were filtered through sterile glass-wool columns before use. Lymphoid cells were also prepared from the lamina propria of the small intestine by an adaptation (Davies & Parrott, in preparation) of a technique used for human colonic mucosa (Bull & Bookman, 1977). Briefly, after washing out the small intestine and excision of Peyer's patches, the epithelial layer was removed by treatment with EDTA and the remaining gut fragments digested with collagenase. Cell suspensions were then purified by isopyknic centrifugation on discontinuous 'gradients' of bovine serum albumin or Ficoll–Triosil cushions. The resultant cell suspensions contained at least 90% lymphoid cells as assessed by Leishman-stained cytocentrifuge preparations. Removal of adherent lymphocytes on nylon-wool columns (Fenwal FT-242) followed a technique previously described (Julius, Simpson & Herzenberg, 1973). Depletion of T lymphocytes was achieved by treatment of cells with a rabbit anti-mouse T cell serum and guinea-pig complement (kindly supplied by Mr John Shields of this department). This antiserum killed 92% of thymus lymphocytes and $< 5\%$ bone marrow lymphocytes when used at the same titre as in these experiments. Control cells were treated with normal rabbit serum and complement.

Cytotoxicity assay. P-815 tumour cells were labelled with sodium ^{51}chromate at 100 μCi/5×10^6 cells/ml for 1 hr at 37°C. Cells were then washed five times and were only diluted to 2×10^5 cells/ml immediately prior to use. Cooke V-bottomed microtitre plates were set up essentially as described by Thorn, Palmer & Manson (1974). Twenty-thousand labelled tumour cells in 0·1 ml of RPMI/NCS were added to 0·1 ml of varying numbers of lymphoid cells or medium. After incubations of 2 hr at 37°C and 1 hr at 45°C to ensure maximal ^{51}chromium release (Burton, Thompson & Warner, 1975), 0·1 ml of the supernatants from the wells was removed and the radioactivity assessed in a gamma counter. Maximum ^{51}chromium release from labelled tumour cells was assessed by adding 0·1 ml 10% Triton X-100 in distilled water to the wells containing tumour cells followed by processing as described previously.

Calculation of results. Data are expressed as the mean per cent specific cytotoxicity of quadruplicate cultures according to the following formula: per cent specific cytotoxicity = (Exp. release − Cont. release/Max. release − Cont. release) × 100. The cells in each experiment were derived from a pool of three mice.

RESULTS

The development of cytotoxic potential in the lamina propria

Up to 5 days after the intraperitoneal injection of P-815 cells there were no detectable cytotoxic responses against P-815 cells in the mesenteric lymph nodes (MLN), spleen, Peyer's patches, bone marrow or lamina propria (data not shown). At day 5, however, there was a very high level of cytotoxicity in the lamina propria (Table 1). At a lymphocyte to target ratio of 50:1, the cytotoxicity in the lamina propria was five times greater than that in the MLN and at least ten times greater than

that in the spleen and Peyer's patches. By day 6, at 50:1, these differentials had almost halved but the actual cytotoxicity in the lamina propria had increased little. Two different experiments are shown in Table 1 for day 6 to indicate the small variability between experiments. In the data for each day, standard deviations of the mean are not shown as these would represent standard deviations between the quadruplicate cultures which are not relevant. In any case, this standard deviation never exceeded 10% of the mean. By days 11 to 13 after injection of P-815 cells, although the levels of cytotoxic cells in the lamina propria had increased slightly compared to day 6, those in the MLN and spleen had risen substantially. The cytotoxicity in Peyer's patches, although increased, was still only half that seen in the lamina propria. The maximum cytotoxic values in each organ appear to have been reached between days 11 and 13. By day 24, values for cytotoxicity in all organs had decreased, the Peyer's patches by the greatest amount and the other organs by half. At a ratio of 50:1, the cytotoxicity in the lamina propria was still at least twice that in any other organ.

The differences between the 100:1 and 50:1 values in the lamina propria at days 6, 11, 13 and 24 should be noted and compared with those in the spleen. At 50:1 in the lamina propria, killing of tumour cells had reached an optimal level and this further reinforced the observation of greater cytotoxic power of lamina propria lymphocytes.

The data from Table 1 has been conveniently summarized in Fig. 1 which shows the pattern of specific cytotoxicity with time after injection of tumour cells using the values obtained from a lymphocyte to target ratio of 50:1. This emphasizes the rapid appearance of cytotoxic cells in the lamina propria.

Characterization of cytotoxic cells

The cytotoxic cells were primarily T lymphocytes (Table 2). After treatment with an anti-T cell serum and complement, no cytotoxic cells remained in spleen, MLN or lamina propria lymphocyte

Table 1. Per cent specific cytotoxicity at various times after i.p. injection of P-815 cells

Day after immunization	Lymphocyte–target ratio	Per cent specific cytotoxicity			
		Spleen	Mesenteric lymph nodes	Peyer's patches	Lamina propria
5	100:1	7·7	14·6	6·3	n.d.
	50:1	3·8	9·8	4·9	48·9
	25:1	3·5	6·5	n.d.	33·2
6 (Expt 1)	50:1	9·3	19·7	5·3	45·0
	25:1	4·8	11·2	2·6	32·6
	12·5:1	2·9	7·5	n.d.	19·9
6 (Expt 2)	100:1	16·0	31·2	n.d.	54·7
	50:1	11·4	21·3	n.d.	56·9
	25:1	6·9	13·8	n.d.	42·9
	12·5:1	3·6	8·2	n.d.	26·8
11	100:1	78·6	57·1	36·4	76·0
	50:1	66·3	50·4	26·4	74·5
	25:1	50·9	36·9	n.d.	62·1
13	100:1	80·7	54·9	35·7	91·6
	50:1	70·2	38·2	25·5	89·9
	25:1	46·6	24·4	n.d.	71·2
24	100:1	42·0	26·6	9·0	48·6
	50:1	26·0	17·1	6·1	48·1
	25:1	17·9	10·0	n.d.	36·8

n.d. = Not done.

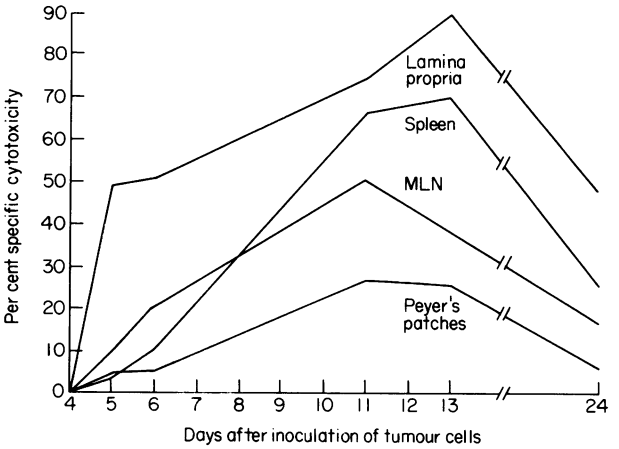

Fig. 1. Comparison of cytotoxicity in various different organs, at a lymphocyte to target ratio of 50: 1, with time after the inoculation of tumour cells.

Table 2. Characterization of cytotoxic cells

Treatment	Lymphocyte–target ratio	Per cent specific cytotoxicity*		
		Spleen	Mesenteric lymph nodes	Lamina propria
Normal rabbit	50:1	8·0	16·4	n.d.
serum plus complement	25:1	6·5	8·7	29·1
	12·5:1	n.d.	n.d.	18·4
Anti-T cell	50:1	0	0	n.d.
serum plus complement	25:1	0	0	0
	12·5:1	n.d.	n.d.	0
Nylon-wool	50:1	11·3	17·6	52·8
filtration	25:1	7·0	11·4	39·1
	12·5:1	4·3	6·5	23·7

n.d. = Not done.
* At days 5–6 after immunization.

suspensions. Depletion of adherent lymphocytes on nylon-wool columns (which also enriched the proportion of T lymphocytes) did not alter the relative proportions of cytotoxicity seen in the spleen, MLN and lamina propria.

H-2 specificity of anti-P-815 response in lamina propria

In order to test the H-2 specificity of cytotoxic lymphocytes in the lamina propria, cytotoxic responses against a number of different tumours were compared (Table 3). High levels of cytotoxicity were seen against the immunizing tumour (P-815) with slightly lower levels against a different tumour with compatible H-2 (L-1210). Cytotoxicity against H-2-incompatible cells, TLX-5 and EL-4, was minimal. Against an H-2^a tumour YAC-1, cytotoxicity was similar to that against L-1210.

Effect of extraction procedure on cytotoxic cells

To test whether the technique of extracting cells from the lamina propria was responsible for the high level of cytotoxicity seen, rather than being a property of the cells *per se*, or indeed in any way

Table 3. H-2 specificity of cytotoxic cells in mesenteric lymph nodes and lamina propria generated against P-815 tumour cells

Tumour type	H-2 specificity	Per cent specific cytotoxicity at lymphocyte:target ratio of 25:1	
		Mesenteric lymph nodes	Lamina propria
P-815	d	7·3	35·0
L-1210	d	4·2	22·7
TLX-5	k	0	3·3
EL-4	b	1·6	1·5
YAC-1	a	3·6	25·7

Table 4. The effect of the extraction technique for lamina propria cells on cytotoxic cells in mesenteric lymph nodes

Lymphocyte–Target ratio	Per cent specific cytotoxocity: MLN cells			
	Stored at:*		After EDTA only†	After EDTA + collagenase
	4°C	37°C		
50:1	12·0	15·7	18·0	19·0
25:1	9·6	10·9	10·5	10·3
12·5:1	4·6	6·7	6·0	7·0

* Storage time: 5–6 hr in complete medium.
† Followed by storage at 4°C for 3–4 hr in complete medium.

affected the cytotoxic properties of lymphocytes, mesenteric lymph node cells were subjected to the same extraction procedures which are normally used on the lamina propria. The two sets of figures on the left-hand side of Table 4 show the effect of the temperature of storage of MLN cells for the duration of the extraction technique (approximately 6 hr in all). Despite very slight differences at a lymphocyte to target cell ratio of 50:1 which are unlikely to be significant, the temperature of storage appeared to have little importance in this system. The remaining data show the effect of the chemicals and enzyme used in the extraction. A whole lymph node was incubated in EDTA for the same duration as used for the gut (about 90 min) and then a cell suspension was made and stored at 4°C before use. The right-hand column shows the effect of EDTA and then collagenase on a whole lymph node. Again, apart from slight differences at 50:1, the extraction methods appeared to have an insignificant effect on cytotoxic cells.

DISCUSSION

The most significant finding of this work is the speed of appearance of a large number of specific cytotoxic T cells in the lamina propria of the small intestine following intraperitoneal immunization. Although cytotoxic responses have been detected at day 4 after intraperitoneal inoculation of tumours in some systems (Brunner *et al.*, 1970), the magnitude of the response was nowhere near as great as that detected here at day 5 in the lamina propria. These high levels remained in the lamina propria for at least 24 days and were always greater than in other organs indicating that the appearance of cytotoxic cells in the gut is not a transient phenomenon. Whether the cytotoxic cell

population detected at day 24 was the same as that detected at day 5 is an interesting point to consider. Either the population is long-lived or divides *in situ* or alternatively is continuously replenished.

It is almost certain that most of the cytotoxic cells in this system are T cells. They are non-adherent to nylon wool and very sensitive to the effects of an anti-T cell serum and complement. It is unlikely that the cytotoxic response is due to NK or K cell populations which, although still poorly characterized, are probably different from T cells (Greenberg *et al.*, 1973; Herberman *et al.*, 1975; Mattes *et al.*, 1979). NK cells have been reported to be insensitive (Kiessling *et al.*, 1976) or slightly sensitive (Herberman *et al.*, 1977) to the effects of anti-Thy 1 serum and complement. P-815 cells are known to be resistant to the action of a population of NK cells as demonstrated in another similar system (Kiessling, Klein & Wigzell, 1975). In our system, we have found (unpublished observation) that the radioactive isotope release from P-815 cells is the same in the presence or absence of a large number of normal spleen cells which readily demonstrate NK activity against an appropriate susceptible cell line (YAC-1). Thus, although we cannot rule out the possibility of some NK activity contributing to the high levels of cytotoxicity in the lamina propria, the greatest response is due to the action of specific cytotoxic T cells.

The cytotoxic cells generated are primarily specific for the H-2 background of the immunizing tumour (see Table 4), and there is some evidence that compatibility of tumour-associated antigens boosts this response. The reaction against a non-H-2-compatible tumour is minimal, also as reported by Bonavida & Bradley (1976) and Green, Ballas & Henney (1978). The cytotoxic response to YAC-1 ($H\text{-}2^a$) cells is interesting; $H\text{-}2^a$ is not an independent haplotype and exhibits both *k* and *d* specificities, the latter of which is recognized by the cytotoxic cells raised against P-815. However, YAC-1 is very sensitive to the effects of NK cells which may further complicate the issue.

The contamination of lamina propria lymphocytes by Peyer's patch cells can be ruled out as an explanation of the cytotoxic cell response. Indeed, the presence of Peyer's patch lymphocytes contaminating lamina propria preparations would presumably reduce the observed cytotoxicity.

The lymphatic drainage of the peritoneal cavity is via the parathymic lymph nodes (Tilney, 1971) and there seems little possibility that, following intraperitoneal immunization, a cellular antigen can activate any of the gut-associated lymphoid system directly. After the intraperitoneal introduction of ovalbumin in Freund's adjuvant, Beh, Husband & Lascelles (1979), examining the origin of antibody-containing cells (ACC) in intestinal lymph of sheep, considered the possibility of direct penetration of the intestinal lymphoid system by the antigen. They suggested that the adjuvant might have increased the permeability of Peyer's patches and/or gut serosa and thus the intestinal ACC might derive from Peyer's patches followed by systemic circulation to the gut or by direct *in situ* stimulation of cells in the lamina propria. In view of the fact that Freund's complete adjuvant causes large numbers of adhesions in the mouse peritoneal cavity (personal observation), the possibility of increased permeability seems likely. However, in our system, the only effect noted in the peritoneal cavity was the temporary growth of the tumour in an ascitic form which was no longer apparent 8 days after the initial injection although foci of solid growth indicated the continuing presence of the tumour. The relative levels of cytotoxic cells in the spleen, Peyer's patches, MLN and gut are inconsistent with any of the current concepts of lymphocyte circulation within the gut circuit (Parrott, 1976) which argues further against the direct penetration theory.

It is possible that the site of injection of the tumour is a key feature. However, Bishop & Donald (1979) have noted that the intravenous injection of P-815 cells results in all the injected cells being killed in the lungs within 6 hr by a non-immunological process. It has also been observed that intraperitoneal or intratracheal instillation of tumour cells results in the presence of cytotoxic T cells in bronchial lymph nodes and pulmonary tissue (Caldwell & Kaltreider, 1978). Alternatively, we must examine the appearance of cytotoxic cells in other mucosal sites like the large intestine and lungs in view of the possibility that all mucosal immune systems have a similar origin (McDermott & Bienenstock, 1979).

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