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The production of hybridomas from the gut associated lymphoid tissue of tumour bearing rats. II. Peripheral intestinal lymph as a source of IgA producing cells

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SUMMARY

Peripheral intestinal lymph afferent to the mesenteric nodes has been collected from rats bearing syngeneic sarcomata in their Peyer's patches and the B cells used to produce rat × rat hybridomas. Analysis of the hybridoma supernatants by radioimmunoassay for the presence of immunoglobulins, showed that hybridomas secreting IgA predominated. Eleven out of the 15 hybridomas selected for antibody binding to cells of the immunising tumour secreted IgA antibodies, and six of these were tumour specific. Efferent mesenteric lymph (i.e. normal thoracic duct lymph), on the other hand, was found to be a poor source of B cells for hybridoma production and no specific IgA secreting hybridomas were obtained. The high yield of IgA secreting hybridomas obtained shows that peripheral intestinal lymph is a better source of IgA committed B cells than are the mesenteric nodes or thoracic duct lymph. We conclude that the IgA producing cells in the latter tissues are too far along the differentiation pathway to plasma cells to undergo successful somatic cell fusion.

Keywords hybridomas gut associated lymphoid tissue IgA tumours

INTRODUCTION

Rats immunized by the injection of allogeneic cells (Hall *et al.*, 1979) or syngeneic tumour cells (Gyure *et al.*, 1980) into the Peyer's patches produce specific IgA antibodies that can be detected in the bile of these animals. We (Dean *et al.*, 1982) have shown that when rats are immunized in this manner with allogeneic cells then hybridomas secreting antibodies of the IgA class can be obtained if cells from the draining mesenteric nodes are fused with the rat myeloma Y3 Ag 1.2.3 (Galfre, Milstein & Wright, 1979). However, further investigation of this system using syngeneic tumours or horseradish peroxidase as antigen (Dean *et al.*, 1984) has shown that although IgA secreting hybridomas can be obtained in this way they are produced less frequently than hybridomas synthesizing IgG antibodies.

It is well established that cells committed to IgA synthesis are derived from the Peyer's patches (Craig & Cebra, 1971; Husband & Gowans, 1978) but relatively little is known about the processes leading to their differentiation *in vivo* into IgA secreting plasma cells. The possibility that mesenteric nodes are not the best source of cells for preparing IgA producing hybridomas led us to investigate the usefulness of cells derived more directly from the Peyer's patches and the lamina propria of the intestine.

When syngeneic tumours were grown in the Peyer's patches of rats, it was not possible to use

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cells of the Peyer's patches for fusion because of the large number of tumour cells present. To circumvent this problem we have obtained samples of peripheral intestinal lymph that would normally drain from the intestine into the mesenteric nodes. Under normal conditions we should expect that this 'afferent' mesenteric lymph would be enriched for B cells switched to sIgA (Kawanishi, Saltzman & Strober, 1983a). In the tumour bearing animals we found that intestinal peripheral lymph was a better source than the mesenteric nodes of cells for the production of IgA secreting hybridomas.

MATERIALS AND METHODS

Animals. Ten week old specific pathogen free male Lister Hooded/Ola rats were taken from our own colony maintained in positive pressure isolators (Isotech, Bicester, UK).

Cell cultures. Hooded and August rat fibrosarcomata and normal cell lines derived from xiphisterna of several rat strains were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) containing fetal bovine serum as described in the preceding paper (Dean *et al.*, 1984).

Inoculation with tumour cells and other surgical procedures. Rats were anaesthetized with ether and the abdomen opened. About 10^6 cultured cells of HSN_{tc} or MC24 in 0·1 ml DMEM were injected into six to eight Peyer's patches. Efferent mesenteric lymph was obtained by cannulation of the 'thoracic duct' (i.e. the cysterna chyli) using standard techniques (Bollman, Cain & Grindley, 1948). The rat was placed in a Bollman restraining cage and lymph was collected at 20–22°C into heparinized DMEM containing 10% fetal bovine serum. Throughout the period of lymph collection the cannulated rats were provided with drinking water containing 0·6% NaCl, 0·05% KCl and 2·5% glucose (Delorme *et al.*, 1969).

In some experiments it was necessary to collect peripheral intestinal lymph, i.e. lymph that had come directly from the intestine and which had not passed through the mesenteric node (afferent mesenteric lymph). It has proved impossible to prepare chronic cannulation of the lymphatics afferent to the mesenteric node so instead the mesenteric nodes were removed and the rats left 4–6 weeks for the lymphatic vessels to regenerate before the animals were challenged with tumour cells. When the thoracic duct of these animals is cannulated it is then possible to collect what is essentially peripheral intestinal lymph that is afferent to the mesenteric node (Hall, Hopkins & Orlans, 1977).

Hybridoma production and screening of supernatants for antibody. Hybridomas were produced as described previously (North *et al.*, 1982) by fusing 5×10^7 cells of the rat myeloma Y3 Ag 1.2.3 with 10^8 lymphoid cells obtained during a 24 h period of thoracic duct drainage. After culturing in HAT selective medium for 6–10 days, cultures were screened for the presence of antibodies that would bind to monolayers of either HSN_{tc} or MC24 using an ^{125}I-labelled anti-globulin binding assay described previously (Hall *et al.*, 1979). With some of the fusion cultures the wells were screened for the presence of immunoglobulins of classes IgA, IgM and IgG using a solid phase radioimmunoassay (Rose, Peppard & Hobbs, 1984).

RESULTS

Collection of intestinal peripheral lymph from rats bearing tumours in the Peyer's patches

Peripheral intestinal lymph, like peripheral lymph from any organ, is distinguished by the presence of dendritic macrophages, which account for 1–10% of all the white cells. In the rat it is a simple matter to remove the mesenteric nodes by blunt dissection without causing significant bleeding. Cannulation of the thoracic duct 4–6 weeks later, when the lymphatics have regenerated, yields what is essentially afferent mesenteric lymph.

When the fibrosarcoma HSN_{tc} was grown in the Peyer's patches of rats, whose mesenteric nodes had been removed, tumour cells were found in the afferent lymph at early stages of tumour growth (< 10 days) and they overgrew the hybridomas in some wells of the fusion cultures. However, as was found when mesenteric nodes were used for fusion, few viable tumour cells were present in the

lymph collected at 18 or 23 days, although the tumours in the Peyer's patches continued to grow. For this reason most fusions were done using lymphocytes obtained at the later times. Tumour cells were not found in the fusion cultures when lymphocytes from either 8 or 18 day efferent mesenteric lymph were used for fusion indicating that the tumour cells were effectively retained by the mesenteric nodes in these animals.

Hybridomas obtained using lymphocytes from afferent or efferent mesenteric lymph

Seven fusions were carried out using lymphocytes from afferent mesenteric lymph collected during a 24 h drainage and a further four fusions were done using lymphocytes from efferent mesenteric lymph. Microscopic examination of efferent lymph from tumour bearers not only confirmed the absence of dendritic macrophages but also showed that compared with afferent lymph fewer blast cells were present. In consequence fewer hydridomas were obtained (Table 1) when efferent lymphocytes were used for fusion.

Fifteen hybridomas, obtained using afferent lymphocytes, secreted antibodies that bound to either HSN_{tc} or MC24 and 11 of these antibodies were of the IgA class. Two of the positive wells were overgrown with HSN_{tc} cells (fusions AL/1 and AL/2) before the specific hybridomas could be isolated and cloned; the isotype of these antibodies was not established. Because of potential problems with overgrowth of wells by contaminating tumour cells we now pick colonies from positive wells as soon as possible, instead of growing them up in the original well. We find this procedure advantageous also when several hybridoma colonies are present in the same well.

In two fusions (AL/2 and AL/4) all of the wells were screened by radioimmunoassay after 21 days growth, for the presence of IgA, IgG or IgM. Table 2 shows that in these fusions, using lymphocytes from afferent mesenteric lymph, the majority of the wells contained IgA secreting hybridomas whereas fewer wells contained IgM or IgG producers.

Reactivity of syngeneic monoclonal antibodies (MoAb) obtained from tumour bearing rats

Seven of the hybridomas derived from afferent lymphocytes produced antibodies specific for the immunizing tumour HSN_{tc}, and six of these were of the IgA class (Table 3). Only one cell binding

Table 1. Hybridomas derived from 'afferent' or efferent mesenteric lymph of rats bearing syngeneic fibrosarcomata

Fusion	Tumour	Days T.B.	Wells +ve hybridomas	Wells +ve sp. antibody	Isotype				
					α	γ2	γ1	μ	other†
Afferent lymph									
AL/1	HSN_{tc}	8	72/96	1*					1
AL/2	HSN_{tc}	18	96/96	6	5				1
AL/3	HSN_{tc}	18	76/96	4	3	1			
AL/4	HSN_{tc}	23	90/96	1	1				
AL/5	HSN_{tc}	23	84/96	2	2				
MCAL/1	MC24	18	56/96	1			1		
MCAL/2	MC24	18	—	0					
Efferent lymph									
EL/1	HSN_{tc}	8	—	0					
EL/2	HSN_{tc}	8	—	0					
EL/3	HSN_{tc}	18	49/96	0					
EL/4	HSN_{tc}	18	59/96	1			1		

* Well overgrown with HSN_{tc} before hybridoma cells could be isolated.

† Isotypes not established.

Table 2. Immunoglobulins secreted by hybridomas derived from intestinal peripheral lymph of rats bearing HSN_{tc}

	% wells containing rat immunoglobulin of class		
Fusion	IgA	IgG	IgM
AL2	95	33	29
AL4	78	3	19

hybridoma was obtained from the four fusions using efferent lymphocytes and this secreted an IgG1 antibody specific for HSN_{tc}. Of the IgA antibodies remaining, one showed a broad tumour reactivity and three were cross-reactive with all rat cells tested that were of fibroblast origin. Only one IgG2 antibody was found (AL/3/12). It was of the IgG2a subclass, specific for HSN_{tc} (Table 4) and was obtained using afferent lymphocytes. Interestingly, only one IgG2a producing hybridoma was obtained using mesenteric nodes (Dean *et al.*, 1984) although the immunoglobulin constitutes the major subclass in serum (Jarrett & Bazin, 1977).

Table 3. Reactivity of syngeneic MoAb derived from 'afferent' or efferent mesenteric lymph of tumour bearing rats

		Reactivity			Total No.
Isotype	HSN_{tc}	MC24	broad tumour	Cross-reactive	hybridomas
Afferent lymph					
IgA	6		1	4	11
IgG2	1				1
IgG1			1		1
Other					2*
Efferent lymph					
IgG1	1				1

* Isotype and reactivity not established.

Table 4. Specificity of selected MoAb derived from 'afferent' or efferent mesenteric lymph of tumour bearing rats (specific ct/min ^{125}I-sheep/rat F[ab']$_2$ bound/well)

Antibody	Isotype	HSN_{tc}	MC24	HSBPA	ASBP1	WAXI	HOXI	NUFLI	FIXI
AL/2/28	α	226	1,005	1,229	1,168	3,853	8,612	1,011	4,425
AL/2/33	α	21,772	21,748	11,183	38,499	5,977	8,147	3,056	6,778
AL/2/95	α	10,731	0	158	0	280	550	0	532
AL/3/12	γ2a	10,407	0	372	167	0	119	0	219
AL/3/64	α	9,006	371	459	133	0	0	0	0
AL/3/86	α	8,808	7,669	3,077	16,983	3,123	2,741	409	1,502
AL/4/89	α	8,840	754	959	1,754	214	136	0	0
MCAL/1/75	γ1	257	4,389	1,950	3,077	305	307	88	502
EL/4/56	γ1	2,629	158	403	432	0	0	0	0

Table 4 shows that the cross-reactive IgA antibodies differed in their cell binding characteristics. For example, while AL/2/33 was totally cross-reactive, AL/2/28 reacted principally with normal cells, whereas AL/3/86 showed a preference for tumour cells. Antibodies reactive with HSN_{tc} alone gave a high degree of specificity (e.g. AL/2/95 and AL/3/12) and currently these antibodies are being investigated for their potential use in tumour localization and adjuvant therapy. Again, as had been found with hybridomas derived from mesenteric nodes (Dean *et al.*, 1984), those antibodies showing broad tumour reactivity gave higher levels of binding to the immunizing tumour (e.g. AL/4/89).

DISCUSSION

One of the main findings of this study was the high proportion of IgA producing hybridomas obtained using cells from afferent mesenteric lymph. Most of the plasma cells in the lamina propria of the rat intestine make IgA (Husband, Monie & Gowans, 1977) so it is not unexpected to find that cells suitable for hybridoma preparation are present in peripheral intestinal lymph. What is surprizing, however, is the relative infrequency of such cells in the mesenteric node (Dean *et al.*, 1984) and particularly in the efferent mesenteric lymph which is the principal component of thoracic duct lymph of normal rats (Husband *et al.*, 1977).

These results may support the view that the mesenteric node is not strictly part of the GALT but like other nodes is concerned largely with the generation of cells that form IgM or IgG and the IgA producing cells which the mesenteric node contains may be a consequence of immigration from the wall of the gut. Even so, the failure to obtain significant numbers of IgA forming hybridomas from efferent lymph was puzzling because it is known that many of the blast cells in this lymph make IgA (Husband *et al.*, 1977; Williams & Gowans, 1975).

One explanation lies in the possibility that the few blast cells present in the efferent lymph were further along the differentiation pathway than their counterparts in afferent lymph and this precluded their successful fusion with the rat myeloma. A similar explanation can be advanced to explain our failure to obtain a higher yield of IgA synthesizing hybridomas from mesenteric nodes (Dean *et al.*, 1984). Certainly, our results are consistent with the findings of others (Kawanishi *et al.*, 1983a, 1983b) that following the switch from sIgM to sIgA production in the Peyer's patches the IgA committed B cells migrate to the mesenteric nodes where they differentiate further. Indeed, specific IgA plaque forming cells can be found in the mesenteric node following injection of antigen into Peyer's patches (Andrew & Hall, 1982).

We are interested in the use of specific IgA antibodies for both tumour therapy and the location of metastatic deposits. In rodents, as in many species, polymeric IgA is rapidly transported from blood to bile (Orlans *et al.*, 1978; Jackson *et al.*, 1978) following binding of the molecules to secretory component present on hepatocyte membranes (Orlans *et al.*, 1979). The rapid clearance of surplus radiolabelled antibodies from the blood pool following intravenous or intra-arterial injection could facilitate the detection of tumour deposits by whole body scanning. This aspect could also be exploited for treatment of tumours when the antibodies are used either alone or conjugated to cytotoxic drugs because in rodents the $T_{\frac{1}{2}}$ of IgA in the blood can be controlled simply by temporary ligation of the bile duct.

Afferent mesenteric lymph is, undoubtedly, a good source of specific IgA committed B cells suitable for hybridoma preparation and we have shown that both individually tumour specific and broadly tumour reactive IgA antibodies can be obtained by growing syngeneic tumours in the Peyer's patches of rats. One problem, also encountered using mesenteric nodes (Dean *et al.*, 1984), was the presence of viable tumour cells in afferent lymph and we are looking at ways of separating the IgA blasts from the tumour cells.

In conclusion, when antigens are injected into the Peyer's patches of rats the GALT provides us with a way to capture separately the antibody producing cells of differing isotype; mesenteric nodes for IgG and IgM producers or peripheral intestinal lymph for IgA producers.

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