Immunology 1994 **81** 643-647

Expression and function of multiple regulators of complement activation in autoimmune thyroid disease

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SUMMARY

Membrane attack complexes of complement occur around thyroid follicles in Graves' disease and Hashimoto's thyroiditis. The lytic potential of such complexes is controlled by membrane-bound and fluid phase regulators and we have investigated the role of these in autoimmune thyroid disease. By immunohistochemical staining, clusterin and S-protein were found in all nine thyroid specimens from patients with Graves' disease and S-protein was found in one of two Hashimoto glands. CD46, CD55 and CD59 were found on thyroid cells in all specimens. CD46 and CD55 expression occurred on thyroid cells cultured *in vitro* and was increased significantly by culture with interleukin-1 (IL-1) and interferon-γ (IFN-γ), which are known to be released by the lymphocytic infiltrate in these conditions. Blocking CD55 had a weak and inconsistent effect on complement-mediated thyroid cell killing *in vitro* but, in four of five experiments, blocking CD46 enhanced killing. However, the effect of blocking CD59 was greater in all cases than blocking CD46 or CD55. Expression of these fluid phase and membrane-bound proteins may be important in determining the severity of thyroid damage produced by complement fixation in Graves' disease and Hashimoto's thyroiditis.

INTRODUCTION

We have previously shown that circulating terminal complement complexes (TCC) are elevated in the serum of patients with Hashimoto's thyroiditis and Graves' disease, falling in the latter condition with treatment, and that thyroid follicles in both disorders are surrounded by TCC, suggesting that active membrane attack complex (MAC) formation is an important feature of autoimmune thyroiditis.[1] Furthermore, thyroid cells are relatively resistant to killing by homologous complement, in part due to the cytokine-regulated expression of CD59 and MAC-inhibitory protein/homologous restriction factor (MIP/HRF), although functionally CD59 appears to be the more important.[2] However, sublethal complement activation impairs the response of thyroid cells to thyroid-stimulating hormone (TSH) and leads to the release of reactive oxygen metabolites, prostaglandin E_2, interleukin-1 (IL-1) and IL-6 and these effects could therefore play a role in the pathogenesis of Graves' disease and Hashimoto's thyroiditis.[3,4]

Other regulators of complement activation besides CD59 and MIP/HRF have not been examined in thyroid disease. Membrane cofactor protein (MCP) or CD46 is a widely expressed cofactor which binds to C3b or C4b, allowing their degradation by factor I.[5] Decay-accelerating factor (DAF) or CD55 is also widely distributed and blocks MAC formation through its action on C3 convertase.[6] As well as these molecules present on target cell membranes, there are at least two important fluid-phase inhibitors of complement activation, S-protein (or vitronectin) and clusterin (or SP-40,40).[7-10] These can bind individually or together to C5b–7 and prevent this complex forming MAC on the membrane.[11] We have therefore examined the intrathyroidal distribution of these four regulators in Graves' disease and Hashimoto's thyroiditis by immunohistochemistry, and performed flow cytometry and functional assays to determine more fully the role of the membrane-bound proteins CD46 and CD55.

Received 14 October 1993; accepted 24 November 1993.

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MATERIALS AND METHODS

Monoclonal antibodies (mAb)

The mAb used were as follows: E5 and G7 (against clusterin) the kind gift of Dr B. Murphy (Melbourne, Australia); GB 24 (against CD46) the kind gift of Dr J. P. Atkinson (St Louis, MO); BRIC 110, 128 and 216 (against CD55) from IBGRL (Elstree, U.K.); MEM 43 (against CD59) the kind gift of Dr V. Horejsi (Prague, Czech Republic) and OX8 (against a rat T-cell subset and used as a control) from Serotec (Bicester, U.K.). Polyclonal rabbit antibodies against clusterin (gift of Dr B. Murphy) and S-protein produced in-house were also used with normal rabbit serum as a control.

Table 1. Distribution of immunohistochemical staining for regulators of complement activation in thyroid sections

	CD46	CD55	CD59	Clusterin	S-protein
Graves' disease ($n=9$)	9 (weak in 3)	9	9	9 (weak in 2)	9 (weak in 1)
Hashimoto's thyroiditis ($n=2$)	2 (both weak)	2 (weak in 1)	2	0	1 (weak)

Immunohistochemistry

Thyroidectomy specimens were obtained from nine patients with Graves' disease (all previously treated with anti-thyroid drugs) and two with Hashimoto's thyroiditis. The specimens were used for immunohistochemical localization on 5-μm cryostat sections essentially as described previously.[1] Briefly, mAb were added to the sections for 60 min followed by a single cycle of anti-mouse IgG for 60 min, alkaline phosphatase anti-alkaline phosphatase for 60 min and then fuchsin substrate (Dako, Glostrup, Denmark). Staining with polyclonal antibodies was developed by addition of a biotinylated goat anti-rabbit IgG and then an avidin-biotin-alkaline phosphatase complex (both from Vector, Burlingame, CA) and finally substrate. Sections were counterstained with Mayer's haematoxylin.

Cell culture and flow cytometry

Thyroid follicular cells were prepared from Graves' thyroidectomy specimens by collagenase/dispase digestion exactly as described elsewhere.[12] Semi-intact follicles were cultured in RPMI-1640 with 10% fetal calf serum (FCS; Flow Laboratories, Irvine, U.K.) for 3 days, with extensive washing to remove non-adherent cells. The thyrocytes were then removed with trypsin and stored in liquid nitrogen until used.

For flow cytometric analysis, the cells were rapidly thawed and cultured at 5×10^5 cells/well of a 24-well plate (Costar, Cambridge, MA) for 3 days with or without the recombinant cytokines IL-1 (Boehringer Mannheim, Lewes, U.K.), tumour necrosis factor (TNF; Biogen, Geneva, Switzerland) and interferon-γ (IFN-γ; Genentech, San Francisco, CA). Cells were removed with phosphate-buffered saline (PBS) containing 10 mM HEPES and EDTA 3 mg/ml, pH 7·4, and gently dispersed by repeated pipetting in PBS with 0·1% (w/v) bovine serum albumin (BSA) and 0·01% (w/v) sodium azide. After incubation with mAb for 60 min at 4°, the cells were washed three times and stained with rabbit anti-mouse/rat IgG–FITC (fluorescein isothiocyanate) conjugate (Sigma, Poole, U.K.). After further washing, flow cytometry was performed on 10^4 cells using a FACScan flow cytometer.

Complement attack on thyroid cells

Cells in suspension were attacked utilizing classical pathway activation essentially as described elsewhere,[2] using a cross-reactivity antibody raised against U937 cells to sensitize the cells. Neutralizing mAb against CD46, CD55 and CD59 were added, as appropriate, with the U937 antibody and the cells incubated for 30 min at 37°. After washing, normal human serum was added at 1:5 dilution, predetermined by initial experiments to give submaximal killing of sensitized thyroid cells, and the cells were incubated for 30 min at 37°. They were washed twice and viability determined by acridine orange/ethidium bromide staining: at least 300 cells were counted with the observer unaware of the sample's prior treatment.

Statistics

Comparison within groups was by paired Student's t-test.

RESULTS

Immunohistochemical staining

The results are summarized in Table 1 and representative staining is shown in Fig. 1. CD46 was observed on thyroid cells in all specimens, although it was weak in some of these. No staining was observed with BRIC 110 but BRIC 128 and BRIC 216 both produced positive staining, indicating the presence of CD55 on thyroid follicular cells. Interstitial tissue was also positive. As shown previously,[2] CD59 was present on all thyroid follicles examined and was located at the apical border of the cells. Perifollicular and interstitial staining was seen equally with the mAb E5 and G7 against clusterin in all of the Graves' specimens but in neither of the Hashimoto glands. The polyclonal antibody against clusterin gave weak staining in only four of the Graves' thyroids. Staining in a similar distribution to E5 was seen with the polyclonal antibody against S-protein, including one of the two Hashimoto patients.

Flow cytometry

Basal expression of CD46 was observed on $17·2\pm3·1\%$ (mean $\pm$SEM) of Graves' thyroid cells prepared from eight individuals with Graves' disease; CD55 was found on $28·0\pm8·0\%$ ($n=6$). Culture with IL-1 100 U/ml increased CD55 expression significantly ($P<0·01$), as judged by an increase in the percentage of cells positive (mean increase $\pm$SEM, $22\pm5·4\%$) and in peak fluorescence channel number: representative results are shown in Table 2 and Fig. 2. IFN-γ at 100 U/ml also increased the percentage of cells positive ($20·3\pm8·3$; $P<0·05$), although in one of the six thyroid cell preparations tested was without effect. CD46 expression was increased by IL-1 ($13·0\pm3·9\%$; $P<0·01$) and there was also a small increase with IFN-γ ($5·1\pm1·9\%$; $P<0·05$). The effect of TNF was tested in one set of thyroid cell cultures and increased CD46 and CD55 expression was observed (Table 2).

Effect of blocking MAC-inhibiting proteins on thyroid cell lysis

The effect of blocking CD46 and CD55 on cell lysis was assessed by incubating the cells with the appropriate mAb prior to complement attack; these results were compared with the effects

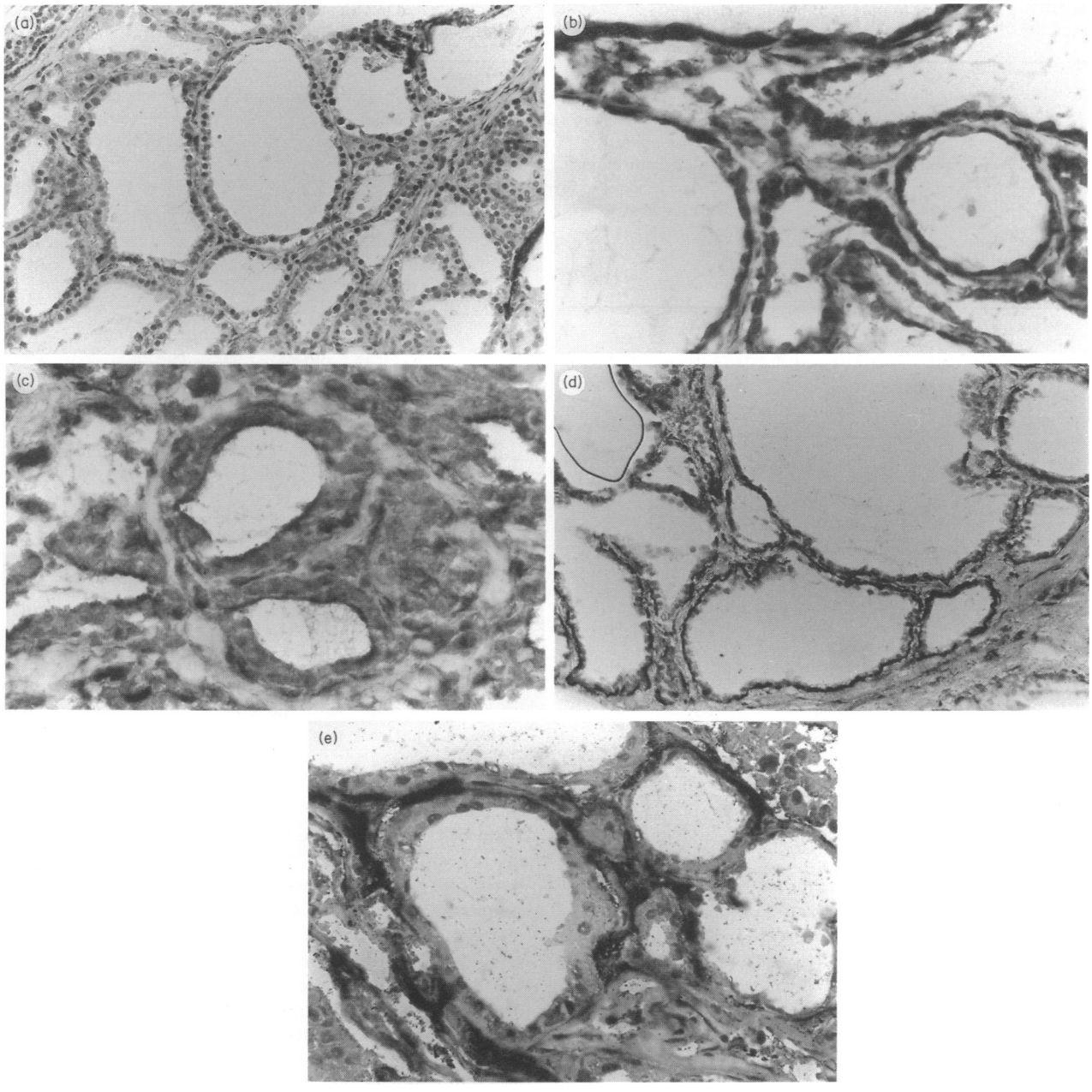

Figure 1. Immunohistochemical staining of thyroid sections from patients with Graves' disease using (a) mAb against CD46, (b) mAb (BRIC 128) against CD55, (c) mAb against CD59, (d) mAb E5 against clusterin, (e) polyclonal antibody against S-protein. Magnification × 100 (a,d), × 200 (b,c,e).

of similarly blocking CD59, and are shown in Table 3. IL-1-stimulated thyroid cells were more resistant to complement-induced lysis: blocking CD59 increased killing of both unstimulated and IL-1-stimulated thyroid cells ($P < 0{\cdot}01$). The effects with blocking CD46 were much less, there being a slight reduction in viability in only three out of the five patient samples tested, and the effect overall for all five unstimulated samples was not significant. There was also no overall significant effect with the addition of mAb against CD55, although killing was increased in four of the five preparations tested, and in patient 1 this effect was particularly clear. Insufficient cells remained from the preparation in which killing was not enhanced to test for CD55 expression.

DISCUSSION

These results demonstrate the presence of soluble and membrane-bound regulators of complement activation in the thyroid in autoimmune thyroid disease, in addition to CD59 and MIP/HRF which we have previously found to be present on the surface of thyroid cells.[2] CD59 was found in the apical region of the thyroid cell membrane, consistent with its targeting to the apical surface by the glycosyl-phosphatidylinositol anchor.[13] Immune complexes, containing immunoglobulins associated with complement deposition, are present on the thyroid basement membrane[14] and TCC, indicating MAC formation, are localized around the thyroid follicles in both Graves' disease

Table 2. Expression of CD46 and CD55 by cultured thyroid cells from a single patient with Graves' disease

	CD46		CD55	
	% cells positive	Peak channel no.*	% cells positive	Peak channel no.*
Nil	14·5	79	58·6	131
IL-1 (100 U/ml)	45·5	141	84·7	144
IFN-γ (100 U/ml)	18·7	80	83·4	147
TNF (10 U/ml)	31·0	142	85·1	144

* Log scale.

Control staining with OX8 mAb gave 0·8–2·1% cells positive, with a peak channel no. of 3–16.

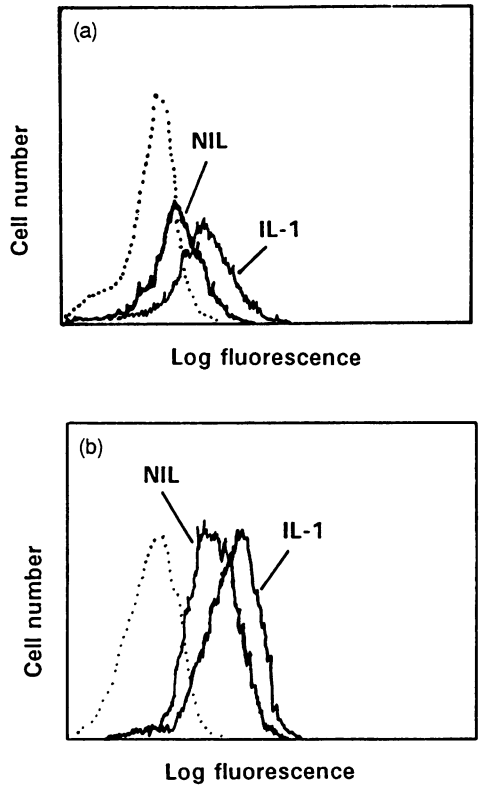

Figure 2. Flow cytometry profiles of thyroid cells from a patient with Graves' disease stained with mAb against (a) CD46 and (b) CD55. Cells were cultured for 3 days alone (NIL) or in the presence of IL-1 (100 U/ml). The dotted line is staining with the control mAb, OX8.

and Hashimoto's thyroiditis.[1] Recently, similar deposition of immunoglobulin and complement have been observed after *in situ* perfusion of rabbit thyroids with homologous sera from animals with experimental autoimmune thyroiditis (EAT), and EAT could be induced in such perfused thyroids by this manoeuvre alone.[15] Furthermore, immunization-induced EAT in rabbits is much less severe in C6-deficient animals.[16] These observations indicate the likely importance of complement-mediated injury in animal and human autoimmune thyroiditis.

Clusterin and S-protein were found in a similar location to TCC[1] in all of the Graves' patients and S-protein was present in one of the two Hashimoto patients. This is in keeping with the frequent co-localization of these two proteins with MAC in human glomerulonephritis, although clusterin in particular is present when MAC deposits contain immunoglobulins.[17] In other examples of MAC deposition, such as in the skin following vascular necrosis, clusterin may be absent.[18] S-protein may also be found in normal skin and kidney[19] and it remains unclear therefore what role this may play in preventing complement activation.

Table 3. Effect of mAb against CD46, CD55 and CD59 on thyroid cells killing by complement

	% cells remaining alive after complement attack			
Patient	Nil	+CD46 mAb	+CD55 mAb	+CD59 mAb
1 NS*	51	37	23	17
S*	82	36	35	16
2 NS	67	53	54	18
S	80	76	70	45
3 NS	38	44	42	11
S	44	54	48	22
4 NS	65	67	60	26
5 NS	59	56	51	24

* NS, non-stimulated—thyroid cells cultured alone for 3 days prior to use; S, stimulated—thyroid cells cultured with IL-1 100 U/ml for 3 days prior to use.

We were unable to demonstrate CD55 by immunohistochemistry using BRIC 110, but staining with the two other mAb against CD55 was positive. Cultured thyroid cells also expressed this molecule, particularly after culture with cytokines known to be released by the inflammatory infiltrate *in vivo*. A wide variety of normal cell types, including gastrointestinal and urogenital tract epithelium, express CD55, and staining is strongest on the more mature epithelial cells in layered epithelium.[20] However, CD55 expression in the thyroid has not previously been documented. CD46 is expressed in almost all tissues and has recently been demonstrated on normal human thyroid cells.[21] We have confirmed this in Graves' disease and Hashimoto's thyroiditis and shown that expression can be enhanced by cytokines.

The functional role of these two proteins was assessed by experiments using mAb to block CD46 and CD55. Only a weak and inconsistent enhancement of complement-mediated killing was observed, although in some individual preparations the effects were more impressive. This is similar to our previous observations with MIP/HRF which had a weak and variable effect *in vitro* in protecting thyroid cells from complement-mediated injury.[2] By contrast, blocking CD59 caused a consistent enhancement of killing, suggesting that this molecule is of key importance in preventing MAC formation on thyroid membranes.

Together, our results show that thyroid cells express at least four regulators of complement activation, CD46, CD55, CD59 and MIP/HRF that can all be enhanced by cytokines and that CD59 appears *in vitro* to be the most important. The soluble complement inhibitors S-protein and clusterin also occur

around the follicles in the same location as we have previously described TCC in autoimmune thyroid disease and may play a role in controlling the lytic potential of MAC in the fluid phase.

ACKNOWLEDGMENTS

This work was supported by the Wellcome Trust. N.T. was in receipt of a Cambridge Nehru Scholarship and Overseas Studentship Award and B.P.M. is a Wellcome Trust Senior Clinical Research Fellow. We are grateful to Mr B. Harrison for provision of the surgical specimens.

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