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Anti-V_H antibodies interfere with antigen binding by human T lymphocytes

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SUMMARY

The effect of antisera against a V_H fragment have been investigated in several T cell proliferative assay systems. Anti-V_H antisera raised in sheep, rabbits and chicken induced profound inhibition of PPD stimulated lymphoproliferation. Likewise were both mixed lymphocyte reaction (MLR) and autologous mixed lymphocyte reaction (AMLR) severely hampered while stimulation induced by mitogens was only minimally affected. Specificity testing indicates that the inhibiting antibodies in these experiments are not directed against native immunoglobulin determinants but rather against determinants specific for the V_H fragment. These results thus support the notion that T cells express V_H antigens and that these antigens are part of or closely associated with the antigen receptor on human T lymphocytes.

INTRODUCTION

The activation of T lymphocytes is still to a large extent an enigma in immunology. Basic information is lacking both of the antigen receptor molecules of these cells as well as the mechanisms by which they recognize and respond to antigens. It appears, however, to be a general feature of T lymphocytes to 'see' antigen in conjunction with MHC antigens on accessory cells. Whether the recognition process involves one receptor binding simultaneously to MHC products and nominal antigen (Doherty *et al.*, 1976; Rosenthal, 1978), or two receptors binding to antigen and MHC respectively (Zinkernagel *et al.*, 1978), is yet an unresolved question. Several lines of evidence suggest the existence of idiotypic immunoglobulin determinants on T cells (Binz & Wigzell, 1977; Rajewsky & Eichmann, 1977; Lea *et al.*, 1979). Thus, genes coding for immunoglobulin variable region determinants may express their products in the T lymphocyte membrane. These reports also raise questions of whether T lymphocytes share a common variable region gene pool with B lymphocytes, or whether these antigen receptors are coded for by a separate set of genes. If derived from a common gene pool, one would expect T lymphocytes to express several variable region markers in addition to idiotypes. In an attempt to investigate this we have raised antisera against immunoglobulin heavy chain variable region (V_H) by immunizing animals from different species with isolated V_H fragments derived from a human IgG3 cryoglobulin. In another paper we have shown that these antisera react with human T lymphocytes probably by V_H specific antibodies (Lea, Michaelsen & Natvig, 1982). In the present report we have investigated how anti-V_H antibodies perturb functional T lymphocyte proliferative assays.

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MATERIALS AND METHODS

Mononuclear cells (MNC). Peripheral blood leucocytes (PBL) were isolated from heparinized normal blood by Isopaque-Ficoll gradient flotation (Böyum, 1976).

Isolation and identification of B and T lymphocytes. A cell population highly enriched in B lymphocytes was isolated by depleting PBL of cells forming rosettes with AET-treated sheep erythrocytes (SRBC) (Pellegrino *et al.*, 1975). B lymphocytes were scored by direct immunofluorescence technique using fluorescein isothiocyanate (FITC) conjugated rabbit anti-human $F(ab')_2$ antiserum (Frøland, Natvig & Stavem, 1972).

T lymphocytes were isolated either by passing PBL through nylon wool columns (Frøland & Natvig, 1973) or by rosetting with AET-treated sheep erythrocytes as above. T lymphocytes were quantitated as cells forming rosettes with SRBC using a common rosette technique (Frøland & Natvig, 1973).

Isolation of immunoglobulin fractions. IgG fractions of human, rabbit and sheep sera were isolated by ion exchange chromatography on DEAE-cellulose columns (DE-52, Whatman Ltd., England) (Michaelsen & Natvig, 1972). Monomers of IgG was obtained by running IgG fractions through calibrated Sephadex G-200 columns. A crude chicken immunoglobulin preparation was isolated by ammonium sulphate precipitation. $F(ab')_2$ fragments of the various IgG preparations were isolated by pepsin digestion (1:100 (w/w), pH 4·0, 18 hr, 37°C) followed by gel filtration on Sephadex G-200 columns. The isolation of the V_H fragment of KUP IgG3 cryoglobulin has been thoroughly described elsewhere (Michaelsen, Førre & Natvig, 1977).

Antisera. Antisera were raised in rabbits, chicken and sheep by injecting the antigens subcutaneously in rabbits and sheep and intramuscularly in chickens. The antigens were emulsified in Freund's complete adjuvant, and the animals were boosted with the same solution 2 weeks after primary immunization and then every month afterwards.

Absorption of antisera. Absorption experiments were performed using columns packed with antigens coupled to Sepharose 4B (Pharmacia, Uppsala, Sweden) either by the CNBr method (March, Parikh & Cuatrecasas, 1974) or to CH- and AH-Sepharose by water soluble carbodiimide according to the manufacturer. The absorptions were performed in PBS while the desorption procedures varied and often included several steps as indicated in each experiment. The desorbed proteins were immediately neutralized with 0·5 M Tris-HCl buffer pH 7·6, dialysed against PBS and concentrated by negative pressure dialysis. During absorptions, the antisera were slowly cycled through the immunosorbent columns overnight, and the columns always contained antigen in large excess compared to the amount of antiserum absorbed. In some experiments the antisera were also absorbed on suspensions of normal human leucocytes or human lymphoid cell lines as indicated in each experiment. In brief, pelleted cells and antisera in a 1:1 volume ratio were incubated for 60 min at 4°C and then 60 min at room temperature before the absorbed antisera were collected after centrifugation. After the absorptions, the antisera were thoroughly dialysed against PBS or Hanks' balanced salt solution in dialysis tubing which was pre-treated by boiling in an EDTA containing solution and extensive washings to detoxify the tubing.

Cell cultures. Triplicate cell cultures were set up in flat bottomed Costar 3596 micro-tissue culture plates. In mitogen or antigen stimulation assays, 10^5 lymphoid cells were used and 20 μl mitogen or antigen solution in a total volume of 120 μl using RPMI 1640 (Flow Lab, Scotland) containing 20% fetal calf serum and antibiotics. Mitogen stimulated cultures were usually harvested on day 3 while antigen stimulation with PPD was terminated after 6 days. Twenty-four hours prior to harvesting 1 μCi ^{3}H-thymidine (Sp. act. 2 Ci/mmol, The Radiochemical Centre, Amersham, England) was added to each culture well.

The allogenic mixed lymphocyte reaction (MLR) was performed using 10^5 responder cells and 10^5 X-ray (2800 rad) or mitomycin C treated stimulator cells in a total volume of 150 μl. MLR cultures were regularly harvested on day 6 after a 24 hr pulse with 1 μCi ^{3}H-thymidine.

Autologous mixed lymphocyte reaction (AMLR) were performed as described for allogeneic MLR except that T cells, isolated by E-RFC depletion of PBL, were used as responder cells and the remaining cells, B lymphocytes and adherent cells, as stimulator cells after irradiation or mitomycin C treatment.

Inhibition experiments were performed by adding antibodies or antibody fragments of the various specificities to the cultures at the beginning of the cultivation period. As controls were used immunoglobulins or Ig fragments from pools of normal sera of the different animal species tested. Viability of the cultured cells was regularly tested at the time of harvesting and was always above 90% as judged by trypan blue exclusion.

RESULTS

Inhibition of mitogen stimulated responses

PBL were cultured in the presence of optimal doses phytohaemaglutinin (PHA), pokeweed mitogen (PWM) and concanavalin A (Con A). As seen on Fig. 1, addition of $F(ab')_2$ fragments of anti-V_H fragment antibodies over a wide concentration range had no or only a modest effect on these responses as measured by ^3H-thymidine incorporation on day 3 after initiation of the cultures. As controls were used $F(ab')_2$ fragments of IgG from normal serum pools and anti-human $F(ab')_2$ antisera.

Inhibition of PPD stimulated responses

PBL were stimulated with purified protein derivative (PPD) in the presence of various antisera. Addition of an IgG fraction of unabsorbed anti-V_H fragment antiserum resulted in a profound decrease in thymidine incorporation reaching background values in some of the experiments (Table 1). The anti-F $(ab')_2$ antiserum also showed significant inhibition although more moderate than the anti-V_H fragment antibodies. Experiments using $F(ab')_2$ fragments of the antisera to avoid problems with lymphocyte activation through Fc receptor interaction gave approximately the same results (Table 1). As PPD is reported to induce polyclonal B cell activation (Nishikawa *et al.*, 1979), the same experiments were carried out with isolated T lymphocytes and irradiated B cells and adherent cells. The inhibition was slightly reduced as compared to experiments with PBL, but T cell proliferation was still strongly affected in this assay (Fig. 2).

Inhibition of allogenic autologous mixed lymphocyte reactions (MLR and AMLR)

The mixed lymphocyte reaction is considered to be a rather selective measure for T lymphocyte function. The same type of inhibition experiments were therefore carried out in one way mixed lymphocyte culture systems using mitomycin C treated or X-ray irradiated stimulator cells. The number of stimulator cells were titrated against the number of responder cells to obtain maximum

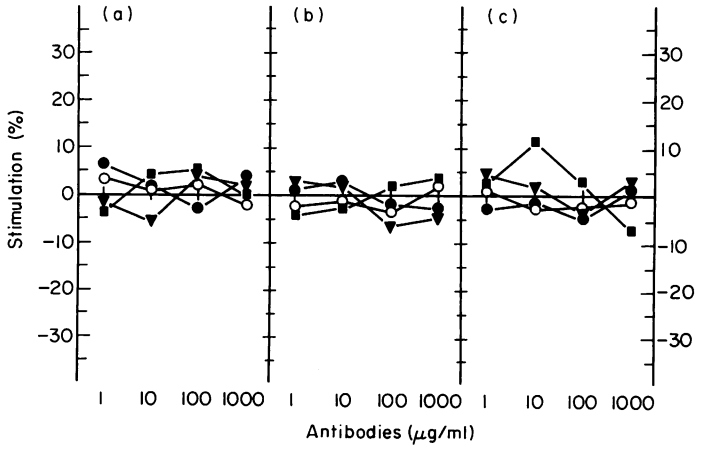

Fig. 1. Effect of different concentrations of S13 anti-V_H antibodies (●), K 564 anti-$F(ab')_2$ antibodies (○), normal sheep IgG (▼) and normal rabbit IgG (■) on lymphoproliferative response induced by the mitogens PHA (a), PWM (b) and Con A (c).

Table 1. Effect of IgG and F(ab′)$_2$ fragments of two different anti-V_H antisera on PPD-induced lymphoproliferation together with anti-F(ab′)$_2$ antibodies and normal immunoglobulins

	PPD response (^{3}H-thymidine uptake, c.p.m.)										
		S 13 anti-V_H		K 631 anti-V_H		K 564 anti-F(ab′)$_2$		Normal sheep		Normal rabbit	
Expt. No.	Control cultures	IgG	F(ab′)$_2$	IgG	F(ab′)$_2$	IgG	F(ab′)$_2$	IgG	F(ab′)$_2$	IgG	F(ab′)$_2$
1	5,185	628	541	772	805	4,030	4,316	6,018	5,104	4,990	5,016
2	2,346	732	865	965	988	1,833	1,974	2,266	2,407	2,216	2,127
3	8,560	467	639	814	857	6,938	7,229	8,691	8,412	8,333	8,429

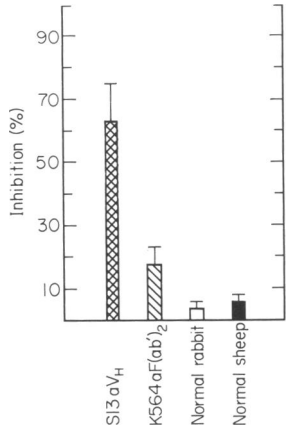

Fig. 2. Effect of F(ab′)$_2$ fragments of anti-V_H antibodies, anti-F(ab′)$_2$ antibodies and normal immunoglobulins on the PPD response in cultures depleted of surface Ig positive cells. Results represent mean and range of five experiments.

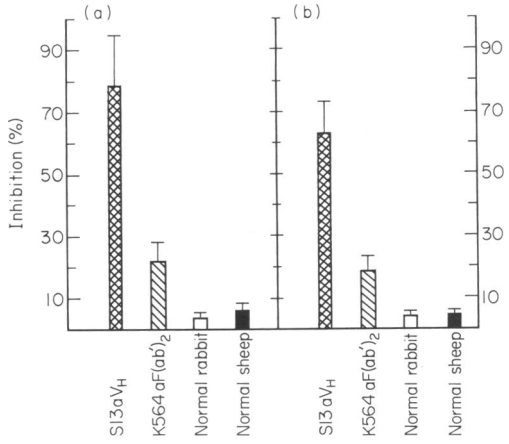

Fig. 3. Effect of F(ab′)$_2$ fragments of anti-V_H antibodies, anti-F(ab′)$_2$ antibodies and controls on MLR (a) and ALMR (b). Mean and range of five experiments are shown.

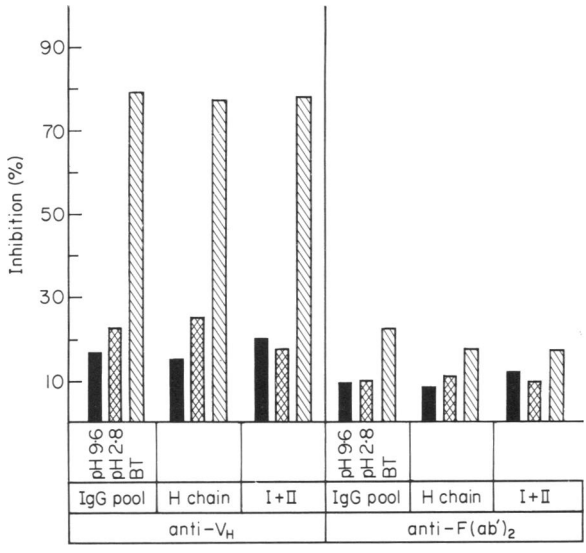

Fig. 4. Effect of various fractions of anti-V_H and anti-F(ab')$_2$ antibodies on PPD-induced responses after absorbing the antibody preparations on immunosorbent columns containing normal human IgG pool or isolated heavy chains. BT represents the break-through fractions, while I + II is the effect of mixed eluates from IgG-coated column and H chain coated column.

inhibition. The results from five different MLR experiments are shown in Fig. 3 concluding that both F(ab')$_2$ fragments of anti-V_H fragment and anti-F(ab')$_2$ antibodies perturb the mixed lymphocyte reaction. In this system, too, the anti-V_H fragment antibodies had much stronger inhibiting capacity than anti-F (ab')$_2$ antibodies, even when immunospecific anti-F (ab')$_2$ antibodies were used. The autologous mixed lymphocyte reaction was also severely hampered by anti-V_H fragment antibodies (Fig. 3b).

The specificity of the antibodies inhibiting PPD induced lymphocyte proliferation

Experiments were designed to characterize the specificity of the antibodies interfering with PPD induced lymphoproliferation. Anti-V_H and anti-F(ab')$_2$ antisera were absorbed on immunosorbent columns containing normal human IgG pool or isolated heavy chains from normal pooled human IgG. The breakthrough fraction and fractions obtained by eluting the immunosorbents successively with 0·1 M $NaHCO_3$ buffer pH 9·6 with 0·5 N NaCl and 0·1 M glycine HCl buffer pH 2·8 were collected and exhaustively dialysed against PBS before testing. The results are shown in Fig. 4, indicating that most of the activity responsible for the inhibiting effect of anti-V_H fragment antibodies on PPD stimulation is found in a fraction not bound to either normal human IgG or isolated heavy chains. However, some inhibition was also found with material eluted from both types of immunosorbents. This was also the case with the anti-F(ab')$_2$ antisera.

Experiments were also undertaken to investigate whether absorptions on different normal human cells or cell lines could remove the inhibiting properties of anti-V_H antibodies in these test systems. Absorptions on human erythrocytes, thrombocytes, the lymphoid and erythroid cell lines RAJI and K562 respectively, chronic lymphocytic leukaemia B cells and the Ig expressing B cell lines U266 and U698 (kindly provided by Dr K. Nilsson, Wallenberg Laboratory, Uppsala) were without effect when tested in the PPD proliferative assay.

DISCUSSION

Lymphoproliferative assays represent sensitive systems for the study of interactions between cells and between antibodies and cells participating in an *in vitro* immune response. Because of the

difficulties with directly demonstrating antigen binding by T lymphocytes, T cell responses have been investigated indirectly by studying the effect of hetero- and allo-antisera in different assay systems. Thus, T cell function have been examined by means of anti-immunoglobulin antibodies (Warner, 1974), anti-idiotype antibodies (Binz & Wigzell, 1977; Rajewsky & Eichmann, 1977) antibodies against the major histocompatibility complex (MHC) especially of the HLA-DR specificity (Schwartz *et al.*, 1976; Bergholtz & Thorsby, 1978) and lately also monoclonal hybridoma antibodies directed at various membrane antigens present on T cells or T cell subpopulations (Chang *et al.*, 1981).

During the last years much effort has been put into the production and characterization of antisera specific for immunoglobulin heavy chain variable regions. This approach to the elucidation of the T lymphocyte receptor structure was initiated by findings that T cell idiotypes cross-reacting with circulating immunoglobulin are coded for by genes linked to the immunoglobulin heavy chain locus (Krammer & Eichmann, 1977). There have also appeared reports claiming the existence of V_H allotypic markers on rabbit T lymphocytes (Cazenave, Cavaillon & Bona, 1977) and that murine T cell derived helper or suppressor factors express V_H antigens in addition to the idiotypic and Ia markers (Bach *et al.*, 1979; Eshar *et al.*, 1980). In accordance with this, it has been reported from the murine system that an anti-V_H antiserum raised against the V_H fragment of the MOPC 315 myeloma protein (Ben-Neriah *et al.*, 1978) react with T lymphocyte receptors by inhibiting antigen binding (Lonai *et al.*, 1978). This antiserum, however, has a very unique reactivity as it does not react with intact immunoglobulins or isolated heavy chains other than the immunizing myeloma protein (Ben-Neriah *et al.*, 1978). This is not the case with the other anti-V_H antisera reported on (Wilder, Yuen & Mage, 1980; Marchalonis *et al.*, 1980). The specificities of the anti-V_H antisera used in the present study are thoroughly described elsewhere (Michaelsen & Lea, 1982), and they all show a considerable reactivity against native immunoglobulins of all isotypes. Although conventional anti-immunoglobulin antisera regularly contain antibodies directed against the heavy chain variable region (Jensenius & Mole, 1978), antisera raised against isolated V_H fragments could have qualitative different properties. Thus, this communication represents the first detailed study of the effect of anti-human V_H fragment antibodies on T cell proliferative assay systems.

Anti-V_H fragment antibodies had little effect on lymphocyte responses induced by the polyclonal activators PHA, PWM and Con A. The different antibodies were tested over a wide concentration range up to 1 mg/ml. These experiments further showed that the anti-V_H fragment antibodies have no stimulating properties *per se* measured by thymidine incorporation, and also that they are not cytotoxic since viability testing by trypan blue exclusion after culture regularly showed more than 90% viable cells.

Stimulation of PBL by purified protein derivative (PPD) is claimed to reflect antigenic triggering of T lymphocytes in humans (Miller & Jones, 1973). When testing anti-V_H fragment antibodies and $F(ab')_2$ fragments of the antibodies in this assay system, a significant inhibition was noted. In fact, in some of the experiments, the PPD response was completely ablated. Anti-$F(ab)_2$ antisera, however, also inhibited the PPD induced response but to a much lower degree. As there have been reports claiming that PPD behave like a polyclonal B cell activator (Nishikawa *et al.*, 1979), some of the effect of anti-V_H as well as anti-$F(ab')_2$ antisera could be ascribed to the inhibition of PPD-induced B cell activation. Control experiments using isolated T cells together with irradiated B lymphocytes and adherent cells indicated a reduced but still significant inhibition by the anti-V_H antibodies.

Another reaction that is thought to depend mainly on T lymphocytes is the mixed lymphocyte reaction (Alm & Peterson, 1970; Andersson, Nordling & Häyry, 1973; Wilson, Silvers & Nowell, 1967). Anti-V_H antibodies strongly perturbed the MLR and AMLR assay systems while anti-$F(ab')_2$ antibodies had a minor but reproducible inhibitory effect. As the AMLR is set up with isolated T lymphocytes and irradiated stimulating B lymphocytes and adherent cells, the inhibition obtained with the anti-V_H fragment antibodies cannot be explained solely by interference with B lymphocyte activation. Indeed, the MLR is also reported to induce immunoglobulin synthesis (Garovoy, Reddish & Abbas, 1979), but our isolated T cell populations contain less than 1% B lymphocytes as judged by several different membrane and enzyme markers (Lea *et al.*, 1979).

In an attempt to delineate the specificity of the antibodies interfering with T cell proliferation, a series of absorption experiments were carried out. Possible heteroactivities were removed by

absorptions on human erythrocytes and thrombocytes. As these extensive precautions were without effect as well as the fact that antisera from several animals within different species showed the same reactivity, these findings provide good evidence for the inhibiting principle in these experiments being a true antibody activity directed against the V_H fragment used for immunization.

Further absorption experiments on immunosorbent columns containing normal IgG pool and isolated heavy chains, failed to remove the inhibiting activity. The strongest inhibition was found in the breakthrough fractions from the immunosorbent columns. Even absorption with several immunoglobulin-expressing B cell lines were without effect on the inhibitory capacity of anti-V_H antibodies. This indicates that the antibody specificities under study is not directed against native immunoglobulin determinants, but rather against V_H specific determinants. Such activities are to be expected in the antisera. It might be relevant to quote the existence of the pepsin agglutinators (Natvig, 1966). These antibodies are directed against determinants exposed only after pepsin digestion of immunoglobulin molecules. Besides, we have results from ELISA inhibition experiments indicating that isolated heavy chains have a better inhibitory capacity than native immunoglobulins when anti-V_H fragment antisera are tested against heavy chain coated tubes (Michaelsen & Lea, 1982). Unfortunately, the V_H fragments are insoluble in all buffers above pH 2·0. It has thus been impossible to perform inhibition and blocking experiments with these fragments. Likewise, investigations on affinity purified antibodies absorbed to and eluted from V_H fragment coupled immunosorbent columns are lacking, both due to shortage of intact myeloma protein and the difficulties encountered in preparing sufficient amounts of V_H fragments completely free of constant region material. However, the most plausible explanation for the results obtained in this study is to anticipate the existence of V_H specific antibodies in the anti-V_H antisera reacting with some V_H-like structures on T lymphocytes. These structures either participate in antigen recognition or are closely associated with structures involved in these processes.

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