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INTERFERENCE OF MONOCLONAL ANTIBODIES WITH PROTEOLYSIS OF ANTIGENS IN CELLULAR AND IN ACELLULAR SYSTEMS

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Summary. - *Monoclonal antibodies complexed with protein antigens can interfere with proteolytic degradation of the antigen. Depending on their fine specificity, they can "protect" well defined regions of the antigen. Because of steric hindrance between bound antibody and proteolytic enzyme, the protected region is larger than the minimal epitope recognized by the antibody binding site. The principle of limited proteolysis proves a valuable tool for the analysis of antigenic fragments and for epitope mapping. Since proteolysis of antigen also occurs in antigen presenting cells, degradation of antigen complexed with a monoclonal antibody (either taken up as an immunocomplex by Fc receptors, or taken up in complexed form with a surface immunoglobulin of a specific B cell) likely modulates the processing pattern of the antigen. This results in a different spectrum of peptides displayed by the antigen presenting cell and thus in different interactions with the antigen specific T cell repertoire. Modulation of T cell response and B cell response by using antigen complexed with different monoclonals is proposed as possible means of interfering with the fine specificity of the response.*

KEY WORDS: monoclonal antibodies, limited proteolysis, modulation of the immune response.

Riassunto (Interferenza di anticorpi monoclonali con proteolisi di antigeni in sistemi cellulari e acellulari). - *Anticorpi monoclonali complessati con antigeni proteici possono interferire con la degradazione proteolitica dell'antigene. In base alla loro fine specificità possono "proteggere" alcune regioni ben definite dell'antigene, e a causa dell'ingombro sterico che si crea tra anticorpo ed enzima proteolitico, la regione protetta di dimensioni maggiori dell'epitopo riconosciuto dal sito antigenico dell'anticorpo. Il principio della proteolisi limitata si è rivelato un valido sistema per l'analisi di frammenti antigenici e per il mappaggio di epitopi. Poiché la proteolisi dell'antigene avviene anche nelle cellule che presentano l'antigene, è possibile modulare la processazione antigenica utilizzando antigeni complessati con anticorpi mnnoclonali. In questo modo si ottiene un ampio spettro di peptidi esposti sulle cellule che presentano l'antigene, e come conseguenza diversi tipi di interazione tra antigene e linfociti T specifici. L'uso di antigene complessato con diversi anticorpi monoclonali si rivelato un buon sistema per modulare la risposta immune di cellule T e B e per interferire con la fine specificità della risposta stessa.*

PAROLE CHIAVE: anticorpi monoclonali, proteolisi limitata, modulazione della risposta immune.

Introduction

Antibodies are known to play an important role as effector molecules of the immune response. In addition regulatory functions of antibodies have been proposed when antibodies were identified as the knots of the idiotypic network [1] and when they were attributed a relevant part in tolerance induction [2]. More recently, since monoclonal antibodies became available, it has been possible to assign to antibodies a previously undetected function, i.e. the capacity to interfere with proteolytic degradation of antigens. This effect has been detected both in cellular and in acellular systems and it will be briefly surveyed here.

Presentation of antigen to T cells

Cellular interactions represent fundamental steps of the immune response. In particular, antibody production is the final event that springs from the interaction of B cells with T helper cells. The concept of antigen bridge as a straightforward pairing between a B cell specific for a B epitope (the hapten) and a T cell specific for a T epitope (the carrier) [3] dwindled when it became clear that T cells only recognise antigen in processed form and presented by antigen presenting cells (APC) in association with class II molecules encoded for by the major histocompatibility

complex (MHC) [4]. APC is a functional definition [5] and in order to fit into this category a cell must fulfill the following requirements:

a) capacity to internalise antigen (uptake);

b) capacity to degrade or process antigen to small peptides (digestion);

c) capacity to associate some of the fragments with MHC class II molecules in the endosomal compartment (association);

d) capacity to display on the surface MHC molecules that bear antigenic fragments (presentation).

Macrophages, monocytes, and dendritic cells are the so called professional APC. They are effective cells for this function, but antigen uptake is random and non selective [6]. Soluble antigen is taken up by constitutive endocytosis and particulate antigen by induced phagocytosis. B cells display a similar antigen presenting capacity [7]. B cells specific for a given antigen (i.e. bearing a surface immunoglobulin of a given fine specificity) offered the clue for a formal explanation of the antigen bridge. Antigen specific B cells can capture the relevant antigen with high efficiency by means of the surface immunoglobulin. Therefore very small amounts of antigen that are missed by the professional APC are taken up, processed and presented only by the antigen specific B cells to specific T cells [8, 9]. T cells recognise processed antigen in the context of MHC II molecules and are in turn induced to secrete lymphokines and proliferate. Some of the lymphokines mediate the helper function of T cells [10]. Therefore, the antigen specific B cell in close proximity with the activated T cells is selectively, but not exclusively, induced to expand clonally and to differentiate in an antibody secreting cell (plasma cell). Obviously these events can take place only if a physical encounter between a specific B cell and a specific T cell occurs.

This is a stochastic event favoured by recirculation of lymphocytes [11]. In a primary response, when the frequency of antigen specific B and T cells is low, chances for encounters are poor. Therefore, clonal expansion of T cells increases the chances for T-B encounters. This is likely due to non specific APC that demand a high antigen concentration. Accordingly, a large amount of antigen is required for promoting a primary response as compared to the relatively small amount of antigen required for boosting.

Preferential pairing or T-B reciprocity

The immune response is usually directed towards protein antigens borne by pathogens that are phylogenetically distant from the responding mammalian species that are being studied. Antigens from viruses, bacteria, fungi, parasites have little homology with the laboratory rodents or with humans, and thus a great deal of different epitopes can be recognised. In contrast, the model antigens such as lysozymes, cytochromes, myoglobins derived from phylogenetically proximal species, that have been extensively used to study the regulation of the immune response [12] display a high degree of homology and a more limited array of potential epitopes. In order to study a protein antigen highly dissimilar from the murine species we chose *E. coli* beta-galactosidase (GZ). The antigenicity of this large tetramer (four subunits of 116 kDa, 1024aa) is well characterized. Antibodies and monoclonals capable of identifying different epitopes on the surface of the native molecule and affecting the enzymatic function of the antigen have been generated [13].

Furthermore, some antibody families protect the catalytic activity of GZ from heat induced denaturation by stabilizing the native conformation. Different antibody families restore the enzymatic activity of GZ molecules produced by mutant strains of *E. coli* bearing point mutations in the Z gene. These antibodies induce the mutant dimers to assemble into wild-type tetramers endowed with enzymatic activity. In addition to different conformational B epitopes, also T epitopes inducing T helper and T suppressor cells were identified on different cyanogen bromide (CB) and tryptic fragments of GZ [14]. Because of the complexity of the antigen, we asked the question whether T helper cells with any fine specificity are equally effective in providing help to any of the B cells available within the GZ specific repertoire. Preferential T-B pairing was demonstrated by showing that only a few CB peptides could prime for T helper cells inducing protecting and activating antibodies, whereas more CB peptides could prime for T cells inducing non activating, non protecting antibodies [15]. These experiments suggest that the fine specificity of the T helper cells can somehow regulate the fine specificity of the antibody response. We subsequently asked how preferential pairings of T-B cells, defined T-B reciprocity by J. Berzofsky [16], occur. We hypothesized that when antigen is taken up by specific B cells via surface immunoglobulins [8], processing of antigen in each B cell is biased by the presence of the complexed immunoglobulin that has been internalised along with antigen. It follows that B cells differing in fine specificity may not display the same processed peptides. Even minor differences may lead to a preferential pairing with a given T cell, or to unfeasable pairing with a cloned T cell if the relevant T epitope is not appropriately displayed on the B cell.

In order to test this possibility, we used an experimental system in which antigen uptake by accessory cells was dependent on the presence of a complexed antibody [17]. Antigen alone at low dose is not internalised, whereas the same dose of antigen in complexed form is avidly taken up via Fc receptors on the APC. In this context antigen processing likely takes place in the presence of the complexed antibody in a system that mimics antigen uptake and processing by the specific B cells that have captured antigen via the surface immunoglobulins. When complexes constructed with different monoclonals were fed to macrophages and used to stimulate a panel of GZ specific T hybridoma clones, two combinations of monoclonal antibody-T clone out of fourty did not function. The same monoclonals were effective to stimulate different T clones

and the same T clones were effectively stimulated with other monoclonal complexes [18]. It follows that there is nothing wrong with either partner (monoclonal - T clone) in the combination and misfiring only results from the particular combination of a given monoclonal with a given T clone. In order to explain this result we hypothesize that processing of antigen complexed with the given monoclonal prevents the appearance of an epitope required for activation of the relevant T clone. In this model it is assumed that monoclonals interfere with antiqen processing. The interpretation of data generated in the GZ system is supported by previous work by Ozaki and Berzofsky [19]. They showed that myoglobin can be internalised by non specific B cells with high efficiency when complexed with a hybrid antibody (a covalent conjugate of anti IgM antibody and anti myoglobin antibody). Also in this system antigen is taken up in complexed form with a monoclonal antibody. T cell activation was reduced with some particular combinations of monoclonal antibody plus T cell clones.

Since the fine specificities of both monoclonal antibody and T cell clones was known, it appeared that the incompatible combination was the one in which both T and B cells recognised closely overlapping epitopes [19].

Different processing patterns in B cells with different fine specificities

The data described strongly supports the hypothesis that antigen processing in B cells is biased by the presence of specific immunoglobulins associated with the internalised antigen present during degradation in the endosamal compartment. Formal evidence for this was provided by Davidson and Watts [20] who made use of antigen specific human lymphoblastoid B cell clones specific for tetanus toxoid (TT). The cells were fed with TT at low dose, therefore antigen could only be internalised via the surface immunoglobulin receptor. Degradation products of iodinated antigen were analysed by SDS gel electrophoresis. Unique patterns of degraded peptides were seen with some B cell clones, clearly indicating that different fine specificities of the antibodies borne by the B cells dictated different proteolytic patterns. Similar experiments are currently in progress in our laboratory: iodinated GZ is complexed with different monoclonals and given to APC (peritoneal macrophages, macrophage lines, B cell lines). Possible differences among the degradation patterns obtained by using different monoclonal antibodies are analysed by SDS gel electrophoresis.

Implications and applications of preferential pairing or T-B reciprocity

The rules that regulate biased processing of antigen complexed with monoclonal antibodies are far from being understood. Several possibilities can be entertained:

a) inhibition of appropriate processing;
b) protection from extensive degradation;
c) interference with peptide/MHC association;
d) interference with re-expression of peptide/MHC complex.

All these possibilities are likely and are not mutually exclusive. Irrespective of the fine mechanisms by which preferential pairings occur, the possibility of manipulating the fine specificities of the immune response exists. In this regard there is a desperate need for effective vaccines against some pathogenesis such as mycobacteria or HIV that induce immune responses easily detected, but not leading to protection. In these representative examples the immune responses may be inappropriate qualitatively, rather than quantitatively. It can be proposed that the new generation vaccines, in addition to purified recombinant antigens, to antigens genetically conjugated with vaccinia virus or the like, to peptide antigens bearing relevant epitopes, should also consider complexes constructed with monoclonal antibodies of different specificities [21]. Masking of a given epitope by monoclonal antibodies, in fact, should prevent uptake by B cells with an identical or overlapping fine specificity. Therefore, antibody responses to epitopes that are dominant, but ineffective for protection, could be amputated.

Hierarchically minor epitopes that are relevant for protection may in turn become dominant. T cell responses can also be modulated in fine specificity as we have shown [18], the same concept of dominant versus recessive and of protective versus non protective epitopes being applied to T cells. The recent development of molecular biology techniques for the generation of single domain antibodies provide additional support to this possibility [22].

Limited proteolysis of antigens complexed with monoclonal antibodies in acellular systems

Monoclonal antibodies have been used as probes to differentiate epitopes on protein antigens. Conversely peptides have been used to identify fine specificities of monoclonal antibodies. Thanks to the remarkable resistance of antigen binding fragments (Fab) to proteolysis, antibodies have also been used for limited proteolysis of antigens.

Eisenberg *et al.* [23] reported the limited proteolysis of a *Herpes simplex* virus glycoprotein induced by V8 protease in the presence to different monoclonal antibodies. SDS gel electrophoresis was used to analyse the peptides obtained by cleavage of free antigen and of complexed antigen. Different patterns of proteolysis by V8 cleavage were obtained depending on the monoclonal used. In particular, a large fragment of 38 kDa remained bound to antibodies in three different groups of monoclonal antibodies.

This suggests that the protective effect is not confined to the epitopes or to its proximity but stretches to a larger region of the antigen, likely because of steric hindrance of the antibody for the proteolytic enzyme.

A similar approach was used with cytochrome c [24]. Peptides released by tryptic digestion of free antigen and antigen bound to different monoclonal antibodies were compared. HPLC was used to separate the peptide mixtures. Unique peptides were detected in the digestion mixtures that contained monoclonal antibodies. Protection was evident either in the presence of intact antibodies or in the presence of their Fab fragments. It was also noted that because of the small size of cytochrome relative to the antibody molecule steric hindrance may be particularly relevant. Thus not all peptides that are delayed in their proteolytic release from complex antigens are necessarily in direct contact with the antibody combining site. Nevertheless, protection was not an all or none phenomenon. If proteolysis was extended for a long period of time, the same peptide patterns were obtained, irrespective of the presence or absence of monoclonal antibodies in the digestion mixture. Measles virus surface glycoproteins were analysed with a similar approach by Sheshberadaran *et al.* [25]. Fine mapping described in this report was defined "protein foot-printing" and was superior to competitive binding assays and good as *in vitro* monoclonal antibody selected variant analysis in differentiating among monoclonal antibodies. The "foot-printing" technique also allowed the detection of conformational changes induced by heat denaturation or by binding of a second antibody to the monoclonal complex.

Deregt *et al.* [26] reported on the mapping of neutralising antigen domains on bovine coronavirus glycoprotein. They used monoclonal antibodies and a panel of different proteolytic enzymes. Complexing with one monoclonal antibody resulted in the preservation of large 50 kDa fragment with identical molecular weight irrespective of the proteolytic enzyme. The same fragment displayed altered mobility in SDS gel under reducing conditions, suggesting the presence of disulphide linkages in the protected fragment. Domains responsible for binding neutralizing antibodies were only present in a large 37 kDa fragment obtained by proteolysis with trypsin. These authors also point out the advantages of epitope mapping by proteolysis of antibody-antigen complexes as compared to conventional Western blotting of proteolytic fragments: a lower amount of antigen is required, antigen for Western blotting is denatured by heating in the presence of SDS and conformational epitopes may be lost, proteolytic cleavage before Western blotting may destroy relevant epitopes that are no longer detectable by antibodies. These drawbacks are not present in limited proteolysis even though it should be kept in mind that protected fragments are much larger than the actual epitopes.

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