Invited Trends Article

Renaissance for Hapten-Specific T Lymphocytes: Implications for Basic and Applied Research

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Protein-reactive chemicals and drugs (haptens), such as dinitro- (DNP) or trinitro-phenylated (TNP) reagents or penicillins, are potent inducers of allergic reactions in experimental animal systems as well as in humans. Hapten-specific T lymphocytes are mediators of these disorders. Like other antigens, hapten determinants on target cells are recognized by the T cell's antigen-specific receptors exclusively in association with gene products of the major histocompatibility complex (MHC). Until recently, the nature of the complex hapten/MHC determinants remained unknown. Atypically, this fact, rather than spurring scientific investigations, caused a rapid loss of interest in haptens as T cell antigens by the majority of immunologists. This situation may now be changing, since we have recently identified MHC-binding, hapten-modified peptides which mediate cellular recognition by T cells *via* their antigen-specific receptors. Synthetic hapten-peptide conjugates open up new routes in studying the molecular details of hapten recognition by T cells. They may also contribute to a better understanding of what defines an antigen as an allergy-inducing "allergen".

Introduction

The immune system represents the major line of defence against microbial infections in vertebrates. It relies on two different, though connected, mechanisms: humoral immune responses mediated by soluble immunoglobulins (antibodies) that are secreted by B lymphocyte-derived plasma cells, and cellular immune responses mediated by thymus-derived T lymphocytes (T cells). The antigen-specific receptor molecules on B and T cells are clonally distributed, and their enormous polymorphism is created by irreversible rearrangement of B and T cell-specific genomic elements (V, D, J) during B und T cell ontogeny [1, 2].

T cells are important in both cell-mediated and humoral immune responses [3]. Their antigen specificity is determined by immunoglobulin-like T cell receptors (TCR), made up of a disulfide-linked glycoprotein heterodimer forming the antigen-binding site [2], and of a complex of several non-polymorphic membrane proteins (the CD3 complex) [4], responsible for signal transmission. Unlike the immunoglobulin receptor of B cells [1], the TCR repertoire is selected during thymic ontogeny to recognize antigens exclusively in the form of small peptides associated with membrane proteins encoded by the major histocompatibility gene complex (MHC class I and class II antigens) [5], a phenomenon termed MHC restriction [6]. The class specificity of MHC restriction is determined by the accessory surface molecules CD8 (class I MHC restriction) or CD4 (class II MHC restriction) [7]. CD8$^+$ T cells usually possess cytotoxic potential (cytotoxic T lymphocytes, CTL) and are essential in the elimination of virus-infected cells [8]. CD4$^+$ cells are normally involved in regulatory processes and therefore termed helper T cells (T$_H$) which mediate their function *via* soluble factors (lymphokines) [9].

Both classes of T cells are, however, not only involved in beneficial defence reactions against microbial infections, but they are also responsible for immunological hyperreactivities such as the various types of allergic disorders [10]. One typical type of allergy-inducing agent (allergen) is represented by low-molecular, chemically reactive, synthetic chemicals or drugs [10]. Although these substances can be bound by specific antibodies, they may elicit immunereactions only after attachment to soluble or cellbound proteins by covalent reaction or complexation. They have therefore been named "half-antigens" or haptens [11].

Due to their chemically defined structure, haptens have played a major part in the elucidation of the specificity and the molecular structure of antibody binding sites [12]. T cells, in contrast, recog-

nize hapten determinants exclusively on hapten-modified cell surfaces, which usually involves a chemical reaction with complete cells [13, 14]. For these reasons, those hapten determinants immunogenic for T cells could not be structurally defined. In contrast to the binding of antigen and antibody, haptens have therefore been of little help in the elucidation of the molecular details of T cell-antigen interactions.

Hapten-specific T cells

In vitro cultures of hapten-specific CTL were first described in 1974 [13]. Shearer and his colleagues showed that class I MHC-restricted trinitrophenyl (TNP)-specific CTL could be induced *in vitro* by stimulation with MHC-congenic cells which had been TNP-modified with trinitrobenzene sulfonic acid (TNBS) (see Fig. 1). Numerous other chemical haptens have later been described to induce hapten-specific CTL [14]. Such T cells can be maintained in permanent cultures (CTL lines) and also cloned from single cells (CTL clones). It has even become possible to produce $CD8^+$ T cell hybridomas by fusion of CTL with variants of the thymoma line BW 5147 [16]. Antigen-specific stimulation of these hybridomas triggers the release of interleukin 2 (IL-2) [16].

Many hapten-specific T cell reactions could be induced in cultures of primary, *i.e.* non-immunized T cells, a phenomenon usually only observed with very strong antigens such as in T cell responses across MHC borders (allo-specific responses). The strength of the TNP-specific T cell responses has, therefore, often been explained by assuming MHC molecules, covalently modified with TNP ("altered self"), to be the major hapten determinants [17].
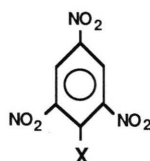
X = Cl   : Picrylchloride
X = SO₃H : TNBS

Fig. 1. Amino-reactive trinitrophenyl derivatives. Picrylchloride in acetone (7%) was used for *in vivo* immunization of mice by skin painting [15]. TNBS (trinitrobenzene sulfonic acid) at 3 mM concentration in PBS was employed for covalent TNP modification of stimulatory or target cells [15].

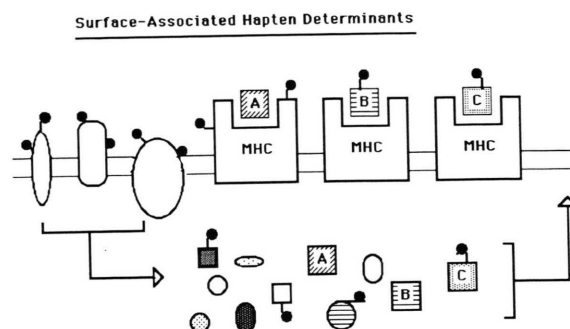Surface-Associated Hapten Determinants

Fig. 2. Possible types of TNP modifications produced by TNBS treatment of intact cells. Black dots represent TNP groups covalently attached to various membrane proteins, including MHC molecules, as well as to different MHC-associated peptides. These MHC-associated TNP peptides may be formed by cellular processing of TNP-modified membrane proteins or by conjugation of TNP to peptides already bound to the specific antigen-binding grooves of MHC molecules.

Nevertheless, CTL reactions against target cells modified with TNP-derivatized soluble proteins have also been described [17–19]. As indicated in Fig. 2, chemical modification of cell surfaces with TNBS may thus lead to an extremely ill-defined multitude of different hapten determinants.

Limited receptor repertoire in TNP-specific CTL

Studies of the TCR repertoire of allo-MHC-specific T cell populations have not revealed an apparent selection for particular receptor elements [20]. This is easily understandable on the basis of the present knowledge on antigen recognition by T cells: a MHC-specific TCR is complementary not only to epitopes of the allo-MHC but also to epitopes formed by the complex of MHC and the multitude of peptides bound to its "binding groove" [21, 22]. We were, therefore, extremely surprised to find the TCR repertoire of MHC ($H-2K^b$)-restricted, TNP-specific CTL in the mouse to be clearly limited: almost half of the CTL clones analyzed were found to have rearranged a member of the $V\alpha 10$ gene family in their TCR α-chain in combination with a $J\beta 2.6$-containing β-chain [15]. Moreover, a defined position at the V-J junctional region of the α-chains (position 93 according to Chothia *et al.* [23]), was always occupied by an acidic amino acid (glutamic (E) or aspartic (D), see Fig. 3). Since even strictly de-

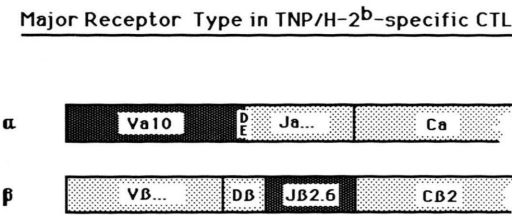Major Receptor Type in TNP/H-2^b-specific CTL

Fig. 3. Predominant receptor elements found in H-2K^b-restricted, TNP-specific CTL. A major part (up to 45%) of in vitro selected, K^b/TNP-specific CTL bear antigen-specific receptors combining Jβ2.6-containing β-chains with α-chains expressing a member of the Vα10 gene family (dark shaded areas). In addition, all α-chains of these types of receptors contained acidic amino acids, i.e. aspartic (D) or glutamic acid (E), at a defined position (amino acid 93) of their Vα-Jα junctional sequences [15].

BSA (222–231)	C A S I Q $\overset{*}{\underline{K}}$ $\underline{F}$ G E R	
MSA (126–135)	C S S M Q $\overset{*}{\underline{K}}$ $\underline{F}$ G E R	
OVA (258–267)	I I N $\underline{F}$ E $\overset{*}{\underline{K}}$ $\underline{L}$ T E W	
K^b binding motif	o o o o $\underline{F}\atop\underline{Y}$ o o $\underline{L}$	

Fig. 4. Synthetic, K^b-associating TNP peptides. Amino acid sequences (single letter code) of 3 synthetic, K^b-binding, TNP-modified peptides, homologous to sequences of bovine (BSA) and mouse serum albumin (MSA) or chicken ovalbumin (OVA). TNP determinants (*) were attached to the ε-aminogroups of lysine (K) by using an ε-N-TNP-lysine derivative for peptide synthesis. Sequences are aligned according to the position of phenylalanine (F) to the postulated motif for K^b-binding peptides. According to Falk et al. [30], naturally processed, K^b-binding peptides are predominantly octamers, containing phenylalanin (F) or tyrosine (Y) in position 5 and leucine (L) in position 8. No constraints were described for the other positions which are indicated by open circles. The BSA- and MSA-derived TNP peptides were recognized cross-reactively by 5 out of 12 independent K^b/TNP-specific CTL. TNP-OVA 258–267 was detected by only 1 clone of this collection which failed to react with the other 2 peptides. None of our clones reacted with the respective TNP-free peptides.

fined antigenic epitopes are known to elicit a spectrum of several "fitting" receptors, we concluded that the number of "T cell relevant" MHC-TNP epitopes on TNBS-modified cell surfaces must be small to allow for a measurable limitation of TCR heterogeneity.

MHC-binding TNP peptides

The apparent limitation of T cell-antigenic TNP determinants encouraged us to attempt the identification of such structures. B. Ortmann in our laboratory [24] has recently succeeded in isolating, identifying and synthesizing TNP-lysine-containing peptides from TNBS-modified soluble proteins such as bovine or mouse serum albumin (BSA, MSA) or chicken ovalbumin (OVA). Three synthetic peptides emerged from these studies (Fig. 4) which could be shown to associate to the class I MHC molecule H-2K^b. Two of these peptides (TNP-BSA 222–231 and TNP-MSA 126–135) efficiently sensitized K^b-expressing target cells for lysis by cytotoxic T cell clones in a completely cross-reactive way. This was the case for 5 out of 12 independently raised CTL clones, specific for K^b-expressing, TNBS-modified target cells. One additional clone selectively recognized TNP-OVA 258–267, whereas 6 clones failed to react with any of the 3 peptides. Thus, the 3 TNP peptides identified TNP epitopes for about 50% of our in vitro selected TNP-specific CTL clones. In this context it is of interest that P. Romagnoli et al. [25] have recently reported that Ni-specific T cells, which occur in Ni-induced contact sensitivities, may be directed against MHC-associated Ni-peptide complexes.
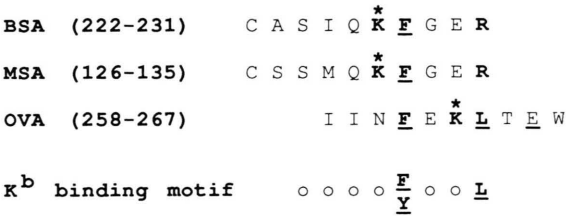
Covalent MHC-TNP determinants are not important

However, for 50% of our clones the target epitopes complementary to their TNP-specific receptors remained unknown. We, therefore, asked the question whether these determinants might be represented by covalently TNP-modified K^b (altered self) molecules.

A. v. Bonin in our group has used the mouse lymphoma RMA-S, a mutant cell line expressing "empty" (peptide-free) K^b-molecules [26, 27], as target cells for a collection of TNP/K^b-specific CTL. He found no evidence for an immunodominance of "altered self" TNP epitopes and clearly demonstrated that some of the clones were even devoid of any reactivity with covalently TNBS-modified RMA-S cells (submitted for publication). MHC molecules, covalently modified with TNP, can therefore, only play a minor role as antigenic determinants for TNP-specific CTL.

Moreover, it could be shown (A. v. Bonin, unpublished results) that several of our CTL clones specific for "unknown" TNP epitopes reacted with target cells sensitized with peptide extracts prepared from TNBS-modified K^b-expressing spleen cells according to the method of H. G. Rammensee and coworkers [22, 28, 29]. This indicates that those CTL clones not reacting with the TNP peptides shown in Fig. 4 are also specific for K^b-associated, though as yet unidentified, TNP peptides.

Role of peptides in determination of TNP epitopes

Recent data from H. G. Rammensee's [30] and other laboratories [31−33] have indicated haplotype-specific restrictions on size and amino acid sequence of naturally MHC-associated peptides. For K^b-associated peptides the minimal structural requirements according to this analysis were as shown at the bottom of Fig. 4: a length of 8 amino acids, a phenylalanine (F) or tyrosine (Y) in position 5, and a leucine (L) in position 8 [30]. S. Martin in our laboratory (data submitted for publication) could demonstrate that trimming of the decapeptide TNP-MSA 126−135 (Fig. 4) to the optimal length of 8 amino acids (128−135) and substitution of the arginine at position 135 by leucine, i.e. full adjustment to the postulated motif of Falk et al. [30], increased the antigenic efficacy of the peptide by a factor of $> 10^4$. This clearly points to the relevance of Rammensee's predictions and allows the directed synthesis of hapten-bearing, K^b-binding peptides.

Indeed, S. Martin found that several synthetic octapeptides obeying the rules for K^b-specificity (F or Y in position 5 and L in position 8) but differing in their remaining amino acid sequences, produced K^b-associated TNP epitopes cross-reactive with TNP-MSA 128−135, provided that they contained a TNP-conjugated lysine in position 4 (Fig. 5A). TNP-lysine in a different position (e.g. position 7, see Fig. 5B) was not recognized by these clones.

These findings infer very clearly that the hapten-bearing peptide mainly provides the function of an "anchor" for the TNP residue in the MHC peptide-binding groove. The amino acid sequence of the peptide anchor is of minor importance in the definition of the hapten epitope. The main function of the peptide sequence is, therefore, to define the position of lysine in the chain. Since lysine is
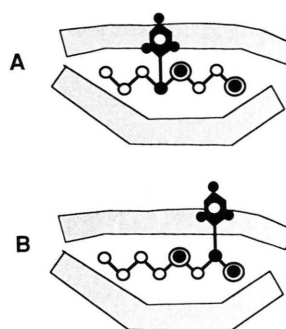

Fig. 5. Schematic representation of different K^b-associated TNP peptides. Helical areas lining the peptide binding groove of the class I MHC molecule (shaded boxes) are arranged according to X-ray refraction data of Bjorkman et al. [34]. The K^b-specific octapeptides are indicated by open circles for undefined amino acids and black, encircled dots for the "anchor" amino acids F(Y) or L in positions 5 and 8, respectively. Black dots, connected to the TNP symbol represent TNP-lysine residues in position 4 (A) or 7 (B).

the only amino acid residue modified by TNBS [35] the positioning of lysine in the peptide directly defines the localization of the hapten-determinant in the TCR-contact-area of the MHC molecule (see Fig. 5).

Conclusions and Outlook

Our findings have several far-reaching implications:

1. The chemical synthesis of high-affinity MHC-binding, hapten-conjugated peptides will allow the production of stimulatory or target cells carrying only one defined type of hapten epitope. This will dismiss the problem of heterogeneous and molecularly undefined antigenic hapten determinants and introduce a new level of structural specificity to studies on hapten-specific T cell systems. The use of defined MHC-anchor peptides will, for example, allow their modification with any desired chemical hapten. This opens the field of TCR hapten interaction to studies on a level of molecular detail comparable to that known for antibody-hapten binding sites. In fact, comparisons of primary TCR structures recognizing or not recognizing a given TNP-peptide/MHC complex have already led to the identification of hapten-contacting TCR regions [36, 37]. Moreover, the new methodology opens the way to using photo-reac-

tive haptens for photo-affinity labelling of antigen contact residues in specific T cell receptors.

2. Another implication concerning basic research on peptide-MHC interactions and the recognition of these complexes by specific T cells is the following: In general, manipulations on the sequence of MHC-binding, antigenic peptides, aiming at alterations of the affinity of the peptide-MHC association, will lead to a simultaneous loss of antigenic specificity. Here we show that within given rules the amino acid sequence of TNP-anchoring peptides can be widely changed without interfering with the TCR-specific TNP epitope.

3. The more practical implications, finally, concern our understanding of allergic disorders. What are the reasons for some selected structures within the universe of antigens to force immuneresponses into the various types of allergic hyperreactivities? Our data may bear on this question in that the nitrophenylated compounds DNP and TNP are among the classical model reagents to induce delayed-type hyperreactivity or contact sensitivity.

TNP may be attached to any MHC-associating, lysine-containing peptide. Moreover, most if not all of these peptides possessing a lysine residue in a defined position of their sequence will give rise to cross-reactive, T cell-specific hapten epitopes. Thus, the number of identical or cross-reactive TNP determinants per cell, *i.e.* the repetitivity of individual epitopes, must be significantly higher for TNP than for the vast majority of "normal" peptide antigens. Future experiments, involving stimulatory cell populations of defined hapten-peptide surface density will have to decide whether this parameter, indeed, may direct immune re-

sponses into "normal" or "hyper" reactivity. The facts that our TNP peptides can be traced with TNP-specific antibodies, and that we can produce cross-reactive TNP peptides of highly varying affinity for MHC are definitely helpful in this respect.

The affinity of peptides for their MHC binding sites may represent a link between protein and hapten allergens. Protein determinants represented by processed peptides of extraordinarily high MHC affinity should become over-represented and produce more repetitive determinants than other peptides on antigen presenting cells.

Given the new technology available for producing class I MHC-associated hapten determinants for T cells (hopefully soon to be applicable also to class II MHC) we feel that many of these questions may now be experimentally answered. This, in turn, should lead to a more detailed understanding of chemically and drug-induced allergic diseases.

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