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In vivo treatment with a monoclonal antibody to T helper cells in experimental autoimmune glomerulonephritis in the BN rat

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SUMMARY

Experimental autoimmune glomerulonephritis (EAG) was induced in brown Norway (BN) rats by a single i.m. injection of homologous glomerular basement membrane (GBM) in Freund's complete adjuvant. This model of anti-GBM disease is characterized by the development, over several weeks, of circulating and deposited anti-GBM antibodies, accompanied by albuminuria. We examined the effects of treatment with MoAb W3/25 (anti-CD4) at different doses, starting at the time of immunization and continued for the duration of the study or for a limited period only. Continued treatment with W3/25, at a dose of 5 or 10 mg/kg intraperitoneally three times per week for 4 weeks, produced a marked reduction in circulating anti-GBM antibodies, absence of detectable deposited antibody and virtual absence of albuminuria. When W3/25 treatment, at 5 or 10 mg/kg, was stopped after 2 weeks, there was still a significant reduction in anti-GBM antibodies and albuminuria at 4 weeks. A similar effect on the disease was achieved when W3/25 was administered only three times during the first week at a dose of 30 mg/kg. Animals injected with W3/25 at a dose of 10 mg/kg through the course of disease showed $< 10\%$ W3/25$^+$ cells by FACS analysis of splenic lymphocytes at week 4, while controls and animals treated for shorter periods showed $> 30\%$ W3/25$^+$ cells. These results demonstrate that W3/25 can prevent the development of EAG, and that this effect is not dependent on persistent depletion of T cells. Further work is necessary to determine whether anti-T cell therapy is effective in established EAG, and may be worth investigating in human anti-GBM disease.

Keywords BN rat glomerular basement membrane experimental autoimmune glomerulonephritis autoimmunity anti-CD4 monoclonal antibody

INTRODUCTION

Goodpasture's syndrome is an autoimmune disease defined by autoantibodies to the glomerular basement membrane (GBM), and characterized by severe glomerulonephritis and lung haemorrhage [1]. The pathogenicity of these antibodies has been demonstrated in passive transfer studies [2], and by the association between circulating antibody concentrations and severity of glomerular injury [3]. The target antigen for anti-GBM antibodies is the non-collagenous domain (NC1) of the $\alpha 3$ chain of type IV collagen, an important structural component of the GBM [4]. Autoantibody production depends upon genetic and environmental factors, and is associated with the MHC class II genes HLA DRw15 (DR2) and DR4 [5]. Currently, treatment of this disease involves rapid removal of circulating anti-GBM antibodies by plasma exchange, together with the use of immunosuppressive drugs [6]. However, such treatment is non-specific and has appreciable side effects. The development of more specific forms of immunotherapy depends upon a better understanding of the mechanisms involved in the induction and regulation of anti-GBM antibody synthesis, which can be approached by the investigation of appropriate experimental models.

Experimental autoimmune glomerulonephritis (EAG), first described in the sheep by Steblay [7], has since been induced in a variety of mammalian species [8,9], including the rat. This has generally required the administration of heterologous renal antigens in Freund's complete adjuvant (FCA) [10,11], or of polyclonal activators [12,13], although our group [14] and others [15] have found that certain rat strains also respond to homologous or isologous GBM. In the model used in this study, brown Norway (BN) rats given a single injection of homologous GBM in FCA developed sustained production of circulating anti-GBM antibodies, linear deposition of IgG on the GBM, albuminuria and mild focal segmental proliferative glomerulonephritis [14]. This model of EAG has many characteristics in common with the human disease, and involves anti-GBM autoantibodies with specificity similar to that of human autoantibodies ([16]; Syrganis *et al.*, unpublished observations). The observation that cyclosporin A can prevent the disease if given

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early, and reduce its severity if introduced later, suggests a major role for T helper cells in the induction of EAG [17]. Further evidence for T cell involvement in EAG is provided by studies in which passive transfer of splenic Th lymphocytes from animals with the disease primed the response of naive recipients to antigenic challenge with GBM [18].

The *in vivo* administration of anti-CD4 MoAbs has proved to be effective in preventing the induction of disease and/or ameliorating the severity of disease in various animal models of autoimmunity. The aim of this study was to investigate the effects of therapy with MoAb W3/25 (anti-CD4) on the development of EAG in the BN rat. The results obtained contribute to our knowledge of the mechanisms underlying the immunopathogenesis of anti-GBM disease, and should help in the design of more specific and effective treatment for Goodpasture's syndrome.

MATERIALS AND METHODS

Experimental animals

Male BN rats, aged 16–20 weeks and weighing 180–250 g, were obtained from our breeding colony established from stock obtained from the Repgo Institute, Rijswick, The Netherlands. All animals were housed in standard conditions and had free access to normal laboratory diet and water.

Preparation of GBM antigen

Collagenase-solubilized GBM was prepared from BN and outbred Sprague Dawley (SD) rat kidneys, as previously described [19]. Briefly, the kidneys were decapsulated, the medulla partly removed, and the cortex forced through sieves in order to isolate the glomeruli. After examination by light microscopy, the glomeruli were disrupted ultrasonically, and the resulting material lyophilized and digested with purified type I collagenase for 1 h at 37°C.

Induction of EAG

EAG was induced in BN rats by a single i.m. injection of collagenase-solubilized SD GBM in an equal volume of FCA, at a dose of 2 mg/kg body weight [14]. Serial blood samples were taken by tail artery puncture under ether anaesthesia, and 24 h urine specimens were obtained by placing animals in metabolic cages.

Assay systems

Radioimmunoassay. Anti-GBM antibodies were measured by a solid-phase radioimmunoassay, as previously described [19]. Briefly, collagenase-digested BN GBM was coated on to microtitre plates (Dynatech) by overnight incubation at 4°C, and test or control sera were applied for 1 h at 37°C. Binding of specific antibody was recognized by incubation with affinity-purified ^{125}I-labelled goat anti-rat IgG (Serotec) for 1 h at 37°C. Results were expressed as percentage binding of known positive pooled sera from $HgCl_2$-injected BN rats.

Rocket immunoelectrophoresis. Urinary albumin concentrations were measured by rocket immunoelectrophoresis [20], using rabbit antisera to rat albumin raised in our laboratory. Results were expressed in mg/24 h.

Renal histology

Direct immunofluorescence. Direct immunofluorescence (IF) studies were carried out by a modification of previously described methods [21], on kidneys obtained at the time of sacrifice 4 weeks after immunization. Briefly, tissue was embedded in OCT II embedding medium (Miles) on cork discs, snap frozen in isopentone (BDH) pre-cooled in liquid nitrogen, and stored at −70°C. Cryostat sections were cut at 5 μm and incubated with FITC-labelled rabbit anti-rat IgG (Serotec). The degree of immunostaining was assessed blind (J.R.) and graded from 0 to 3+.

Light microscopy. Renal tissue, taken at the time of sacrifice, was fixed in 10% neutral buffered formalin, processed and embedded in paraffin wax for light microscopy by standard techniques. Sections (3 μm) were stained with haematoxylin and eosin and periodic acid-Schiff.

Production, purification and characterization of monoclonal antibody W3/25

The antibody-producing murine hybridoma W3/25 was purchased from Serotec Ltd. This antibody reacts with the rat counterpart to the L3T4 or CD4 antigen which is present on a T helper cell subset and on certain macrophages [22]. The cells were grown in RPMI 1640 medium, supplemented with 5% fetal calf serum (FCS), 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM L-glutamine. Supernatants were harvested by centrifugation at 10000 ***g*** and the immunoglobulin fraction enriched by precipitation with 18% sodium sulphate. The MoAb preparation was dialysed against PBS, total IgG content determined by spectrophotometry at 280 nm, and sterile filtration performed before injection. The specificity of the antibody was confirmed by labelling normal spleen cells, and comparing the FACS profile with that of commercially available W3/25 ascites fluid (Serotec).

FACS analysis

Spleen cells were labelled with MoAb MRC W3/25 (Serotec) by previously described methods [23]. Briefly, 1×10^5 spleen cells were washed three times in PBS, and incubated with 20 μl of 1/100 MoAb in RPMI 1640 medium (supplemented with 5% FCS) for 30 min at room temperature. After further washing with PBS, labelled cells were detected by incubation with microfuged FITC sheep anti-mouse IgG in 20% normal rat serum/PBS for 20 min at room temperature. Cells were then washed with PBS, and erythrocytes lysed by suspending the pellet in distilled water for a few seconds, followed immediately by adding an equal volume of ×2 PBS. The cells were washed with PBS, fixed in 1% paraformaldehyde in PBS, and kept in the dark until analysis on a Coulter EPICS fluorescence-activated cell sorter. Labelled cells were expressed as a percentage of total spleen cells, after subtraction of a negative control. Initially, negative controls were labelled with a mouse MoAb (mouse anti-human GBM), which did not cross react with rat lymphocytes, but this was found to give results similar to controls incubated with medium.

Experimental design

Results from pilot experiments showed that W3/25 at 10 mg/kg three times per week was effective in EAG, in comparison with a control MoAb of the same IgG subclass (mouse anti-human Thy-1, kindly donated by Professor M. Ritter, Royal Postgra-

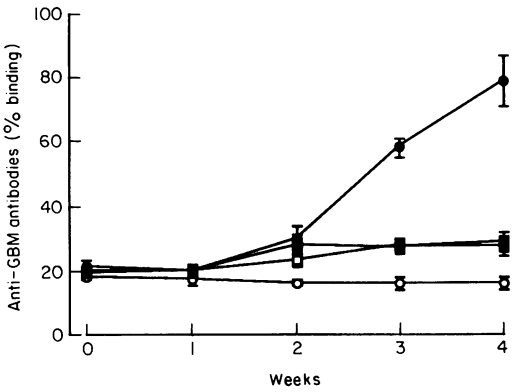

Fig. 1. Effect of continous W3/25 treatment at 5 or 10 mg/kg on anti-glomerular basement membrane (GBM) antibodies in brown Norway (BN) rats ($n=5$) immunized with GBM in Freund's complete adjuvant (FCA). Results shown represent the mean ± s.d. in groups of animals treated with saline (●), W3/25 5 mg/kg (□), W3/25 10 mg/kg (■), and control animals given FCA alone (○).

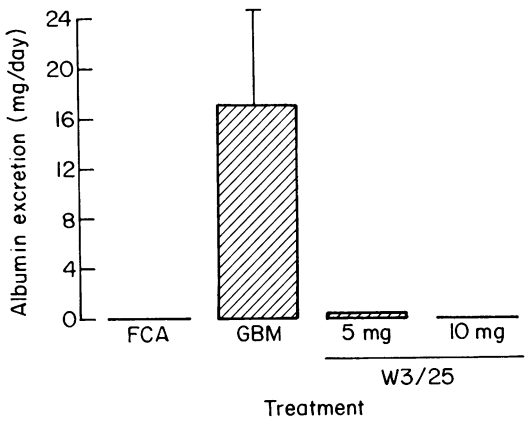

Fig. 2. Effect of continuous W3/25 treatment at 5 or 10 mg/kg on albuminuria in brown Norway (BN) rats ($n=5$) immunized with glomerular basement membrane (GBM) in Freund's complete adjuvant (FCA). Results shown represent the mean ± s.d. in groups of animals treated with different doses of W3/25 at week 4 after immunization.

duate Medical School). In subsequent experiments comparing different treatment regimens, saline was used as the treatment control.

Groups of animals ($n=4$–6), immunized with SD GBM in FCA, were given i.p. injections of: (i) W3/25 at a dose of 5 or 10 mg/kg, three times per week for 4 weeks (the duration of the experiment); (ii) W3/25 at a dose of 5 or 10 mg/kg, three times per week for 2 weeks, then stopped; (iii) W3/25 at a dose of 30 mg/kg, three times per week for 1 week, then stopped; (iv) saline, three times per week for 4 weeks.

Control animals given FCA alone received either: (i) W3/25 at a dose of 10 mg/kg, three times per week for 4 weeks; or (ii) saline, three times per week for 4 weeks.

Statistical analysis

Differences between data were determined by the two-sample Student's *t*-test, and by the Mann–Whitney *U*-test when comparing smaller groups ($n<5$). Analysis of variance was used to confirm differences between multiple data.

RESULTS

Effect of continued treatment with monoclonal antibody W3/25

Anti-GBM antibodies. All control rats injected with GBM in FCA produced detectable circulating anti-GBM antibody, levels of which rose between week 0 and week 2 and peaked at week 4. W3/25 at a dose of 5 or 10 mg/kg, three times per week for 4 weeks, significantly reduced circulating anti-GBM antibody concentrations ($P<0.001$) to levels close to those in controls given FCA alone (Fig. 1).

Albuminuria. All control rats immunized with GBM in FCA produced low but significant levels of urinary albumin by week 4. W3/25 at a dose of 5 or 10 mg/kg reduced albumin excretion to nearly undetectable levels ($P<0.001$). Control animals given FCA alone did not develop albuminuria (Fig. 2).

Direct immunofluorescence. Direct IF of kidney tissue at 4 weeks revealed that control animals given GBM in FCA showed strong linear fluorescence for IgG along the GBM. Animals treated with W3/25 at 5 or 10 mg/kg had no detectable fluorescence. Control animals given FCA alone showed no antibody binding. Results are summarized in Table 1 and illustrated in Fig. 3.

Light microscopy. None of the animals showed significant proliferative glomerulonephritis or interstitial damage. Absence of histological abnormalities at week 4 was not unexpected, since we have previously found such changes to be mild and variable even at 6 weeks [14,17].

FACS analysis of splenic lymphocytes. Normal and positive control animals showed > 30% $W3/25^+$ cells in a suspension of splenic lymphocytes at week 4, while animals given continuous treatment with W3/25 at a dose of 10 mg/kg, three times per week, showed < 10% $W3/25^+$ spleen cells. Results are summarized in Table 2.

Effect of limited treatment with monoclonal antibody W3/25

Anti-GBM antibodies. When W3/25 treatment at a dose of 5 or 10 mg/kg was stopped at week 2 after immunization, anti-GBM antibody levels were reduced at both week 3 ($P<0.001$)

Table 1. Effect of W3/25 treatment on deposition of IgG on the glomerular basement membrane (GBM) in brown Norway (BN) rats with experimental autoimmune glomerulonephritis (EAG)

	3+	2+	1+	−
Four weeks				
Saline	5	0	0	0
5 mg/kg	0	0	1	4
10 mg/kg	0	0	0	5
Two weeks				
5 mg/kg	0	1	3	1
10 mg/kg	0	2	3	0
One week				
30 mg/kg	0	0	3	2

Results are expressed as intensity of fluorescence at week 4 for individual animals.

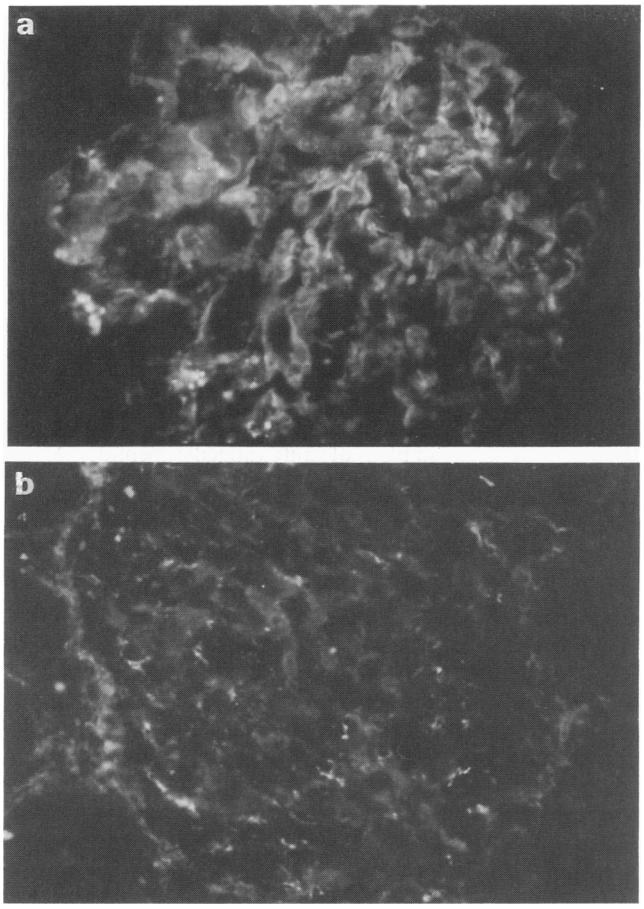

Fig. 3. Direct immunofluorescence of kidney tissue at week 4 in brown Norway (BN) rats immunized with glomerular basement membrane (GBM) in Freund's complete adjuvant (FCA) showing; (a) linear deposition of IgG along the GBM in an animal treated with a control MoAb; (b) negative findings in an animal treated continuously with W3/25 at 10 mg/kg.

and week 4 ($P < 0{\cdot}005$), but were still significantly higher at week 4 than in controls given FCA alone ($P < 0{\cdot}05$) (Fig. 4a). When a high dose (30 mg/kg) of W3/25 was given for the first week only, no significant anti-GBM antibody production was observed by week 3 compared with immunized controls ($P < 0{\cdot}001$), while a small but insignificant rise was observed by week 4 (Fig. 4b).

Albuminuria. Urinary albumin excretion at week 4 in rats given W3/25 at 5 or 10 mg/kg, three times per week for 2 weeks, or at a dose of 30 mg/kg for the first week only (Fig. 5), was reduced to undetectable levels compared with immunized controls ($P < 0{\cdot}001$).

Direct immunofluorescence. Renal tissue at week 4 from animals treated with W3/25 at 5 or 10 mg/kg for 2 weeks showed moderate to weak linear fluorescence for IgG on the GBM. Similarly, those given W3/25 at 30 mg/kg for 1 week showed only weak fluorescence. Results are summarized in Table 1.

Light microscopy. No significant changes were seen.

FACS analysis of splenic lymphocytes. When W3/25 was administered for a limited period, either 2 weeks at a dose of 10 mg/kg or 1 week at a dose of 30 mg/kg, W3/25$^+$ cell numbers in the spleen at week 4 were similar to those in positive and normal control animals. Results are summarized in Table 2.

Table 2. Effect of W3/25 treatment on splenic T helper cell numbers in brown Norway (BN) rats with experimental autoimmune glomerulonephritis (EAG)

	% W3/25$^+$ spleen cells
Saline/4 weeks	32+3
10 mg/kg per 4 weeks	9+4*
10 mg/kg per 2 weeks	26+5
30 mg/kg per 1 week	29+8
FCA/saline per 4 weeks	30+2

Results shown represent the mean+s.d. in groups of animals treated for different periods of time (* $P < 0{\cdot}001$).
FCA, Freund's complete adjuvant.

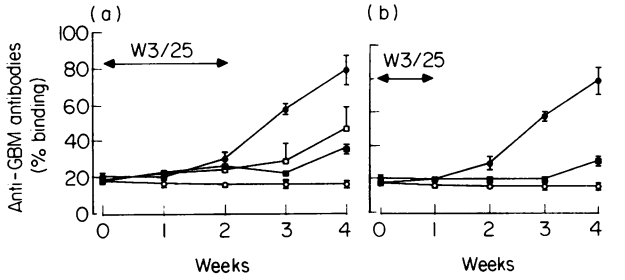

Fig. 4. Effect of W3/25 on anti-glomerular basement membrane (GBM) antibodies in brown Norway (BN) rats ($n = 4$–6) immunized with GBM in Freund's complete adjuvant (FCA) and treated for: (a) 2 weeks at 5 or 10 mg/kg; (b) 1 week at 30 mg/kg. Results shown represent the mean ± s.d. in groups of animals treated with saline (●), W3/25 5 mg/kg (□), W3/25 10 mg/kg (■), W3/25 30 mg/kg (□), and control animals given FCA alone (○).

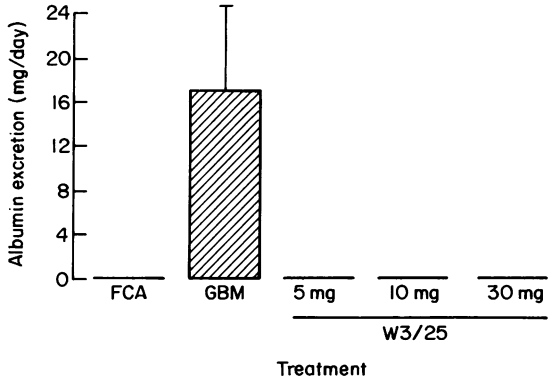

Fig. 5. Effect of W3/25 on albuminuria in brown Norway (BN) rats ($n = 4$–6) immunized with glomerular basement membrane (GBM) in Freund's complete adjuvant (FCA) and treated for 2 weeks at 5 or 10 mg/kg or 1 week at 30 mg/kg. Results shown represent the mean ± s.d. in groups of animals treated with different doses of W3/25 at week 4 after immunization.

DISCUSSION

In order to gain insight into the induction and regulation of the autoimmune response in Goodpasture's syndrome, we have developed an animal model of EAG in the BN rat. This model is induced by immunization with collagenase-digested homologous GBM in FCA [14], and avoids the use of heterologous antigens [10] or polyclonal activators [12], which could lead to a broader immune response than is seen in the human disease. We now have evidence that the autoimmune response in EAG is directed towards the same, or a very similar, antigenic target as that in Goodpasture's syndrome. Autoantibodies from both species bind to the same selected basement membranes in kidney and lung on immunohistology [14,21], and recognize cationic monomers and dimers of the NC1 domain of type IV collagen on 1-D and 2-D Western blotting (Syrganis *et al.*, unpublished observations). There is also increasing evidence for the role of T lymphocytes in the induction of EAG. Cyclosporin A is effective in prevention and treatment [17], and T helper cells from rats with EAG prime naive recipients for development of disease [18].

Previous work has demonstrated the effectiveness of W3/25 in the prevention and treatment of various forms of experimental autoimmune disease in rats. In streptococcal cell wall-induced arthritis, treatment with anti-CD4 MoAb before the onset of disease induced resistance to arthritis, while treatment during ongoing disease resulted in amelioration of arthritis [24]. In autoimmune diabetes mellitus (IDDM), anti-CD4 MoAb prevented spontaneous development of disease, and reversed diabetes if introduced later [25]. Similarly, in experimental autoimmune encephalomyelitis (EAE), treatment with anti-CD4 MoAb prevented development of neurological disease, and also reversed EAE when given to paralysed animals [26]. Treatment with anti-T cell MoAb has also prevented the development of disease in experimental autoimmune thyroiditis [27], experimental autoimmune neuritis (EAN) [28] and experimental autoimmune sialadenitis [29]. MoAbs specifically directed against the α/β TCR have also been reported to be effective in the treatment of T cell-mediated autoimmune diseases in the rat, including EAE [30], EAN [31] and adjuvant arthritis [32].

The present study shows that W3/25 can inhibit the development of EAG in the BN rat if given throughout the course of the disease, or for only a limited period. Continued treatment was effective in suppressing manifestations of disease at doses of 5 or 10 mg/kg body weight, whereas when treatment at similar doses was stopped after 2 weeks there was a slight increase in circulating and deposited anti-GBM antibodies by week 4, but no increase in albuminuria. This implies that not all autoreactive T cells were depleted or anergized when anti-CD4 antibody was given at these doses for a limited period. It also raises the question as to whether antibody deposition alone is sufficient to cause proteinuria—although the amount deposited in these animals may have been subnephritogenic. When W3/25 was given at a high dose of 30 mg/kg for the first week only, no significant antibody production or renal injury was observed by week 4. This suggests that tolerance to the GBM antigen may have been induced by high dose W3/25, and this question could be addressed by rechallenge experiments [24]. This idea is supported by the observation that animals given W3/25 at a dose of 10 mg/kg through the course of the disease showed $< 10\%$ W3/25$^+$ cells in the spleen at week 4, while groups treated for a shorter period of time showed $> 30\%$ W3/25$^+$ cells, even though EAG was inhibited in these animals. Since T cell depletion was not necessary for the inhibitory effect of W3/25, then presumably autoreactive Th cells were anergized or suppressor mechanisms were induced. Similarly, treatment with anti-CD4 MoAb has proven effective in re-establishing self tolerance in spontaneous IDDM in the NOD mouse [33].

In conclusion, we have shown that anti-CD4 MoAb therapy is effective in the prevention of EAG in the BN rat, when administered throughout the course of the disease or for a limited period. Our results imply that EAG results from a T cell-dependent autoimmune response, which is susceptible to immune intervention directed against T helper cells. This model of Goodpasture's disease should be suitable for the investigation of more specific forms of immunotherapy directed against the trimolecular complex of autoantigenic peptide, MHC molecule and T cell receptor [34,35].

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