Heat shock proteins in autoimmune disease. From causative antigen to specific therapy?

Dedicated to Professor Hermann A. Moser on the occasion of his 71st birthday.

X.-D. Yang[a] and U. Feige[b]

[a] *Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford (California 94305, USA), and* [b] *Department of Inflammation, Pharmaceuticals Research Division, Ciba-Geigy, Basel (Switzerland)*

Abstract. Heat shock proteins (hsp) are highly conserved from bacteria to man. Bacterial hsp, with approximate molecular weights of 60 kDa (hsp60), are immunodominant antigens that are immunologically cross-reactive with their mammalian counterparts. Hsp molecules are therefore useful in studies of fundamental questions concerning immune responses to foreign as opposed to self antigens. The finding that immune responses to hsp are associated with both experimentally-induced and spontaneous autoimmune diseases in animals has prompted intensive research to assess the role of bacterial hsp as the etiological agents involved in the development of autoimmune diseases. Recent evidence from animal models of autoimmune disease has clearly demonstrated the involvement of hsp in both the pathogenesis and the immunoregulation of autoimmune diseases. Studies with arthritogenic and diabetogenic T cell clones have identified immunogenic epitopes of hsp. These have been shown to ameliorate adjuvant arthritis in Lewis rats, and insulin-dependent diabetes mellitus (IDDM) in non-obese diabetic (NOD) mice. Such studies may have important therapeutic implications for the future treatment of human autoimmune disease.

Key words. Heat shock protein; autoimmune disease; peptide vaccination; immunotherapy; autoantigen; adjuvant arthritis; insulin-dependent diabetes mellitus; NOD mouse.

Introduction

The genes of heat shock proteins (hsp) were first recognized by a new puffing pattern induced by heat shock in chromosomes of *Drosophila bucksii*[57]. Initially, hsp were classified into families by molecular weight[42], and the utility of this system of classification of hsp is supported by more recent DNA and protein sequence data. It is evident that hsp are highly conserved from prokaryotes to eukaryotes[30,49], which is consistent with their having an essential role in cell survival[49,34,47]. Most of our discussion in this review will concentrate on hsp60, the 60-kDa family members. We will use the following designations for members of the hsp60 family: Mb-hsp60/65 for the 65-kDa hsp60 of *Mycobacterium bovis*, GroEL for hsp60 of *E. coli*, and h-hsp60 for human and m-hsp60 for mouse hsp60.

A number of observations in different fields of research indicate a role for hsp in the development of autoimmunity in general[8,29,35,41,48] and of autoimmune disease in particular (see table 1). This is perhaps surprising, since hsp are highly conserved proteins and might be expected to be treated as "self" by the immune system of mammals. However, as described by Kaufmann[35] and Mollenhauer and Schulmeister[45] in this Multi-author Review, immune responses to hsp are readily detectable not only in disease states, but also in the normal, healthy state. Thus, hsp60 represent a predominant class of highly immunogenic antigens (formerly called 'common antigen') of bacteria[77]. In addition, recent evidence suggests that hsp65 may provide a link between bacterial infection and the development of autoimmunity or autoimmune disease, as recently reviewed[8,9,11,19,34,36,38–40,66,71,76,78].

However, many questions remain regarding the role of hsp in autoimmunity and autoimmune disease. In this article we will summarize what is known about the role of hsp in autoimmune disease and the prospects for the use of hsp in immunotherapy of autoimmune diseases.

Hsp and autoimmune disease

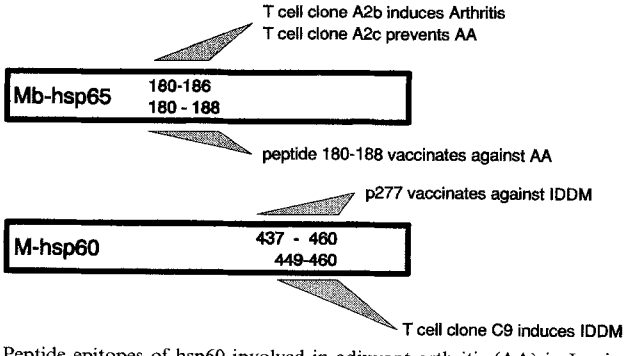
Adjuvant arthritis (AA) can be induced in susceptible strains of rats by injection of a suspension of heat-killed, ground mycobacteria in oil[51]. Disease can be transferred to healthy animals by T cells taken from rats with AA[24,52]. T cells can be maintained in vitro as lines or clones by biweekly cycles of stimulation with antigen and antigen-presenting cells, followed by an interleukin-2 driven expansion of activated T cells. In this way the T cell line A2 was grown from lymph node cells of rats immunized with mycobacteria, and it was shown to induce arthritis when transferred back into irradiated rats[24]. After cloning of A2, two T cell clones were isolated that either induced arthritis (clone A2b), or protected against the development of AA (clone A2c)[12,25,66]. Using these T cell clones Cohen, van Eden and colleagues demonstrated that Mb-hsp60/65 is the mycobacterial antigen involved in AA in rats[68]. The epitope recognized by both T cell clones was mapped and shown to be located in peptide 180–188[68]. It was more precisely localized later, to peptide 180–186 of Mb-hsp60/65[65] (fig. 1).

After the identification of hsp60/65 as the antigen for T cell clones A2b and A2c, it was demonstrated that rats could be protected against AA by pretreatment with Mb-hsp60/65[5,68]. Further studies showed that Mb-hsp60/65 vaccination prevents or modulates disease in a number of experimental arthritis models (reviewed by Feige and Cohen[19]); for example in streptococcal cell wall (SCW)-

Table 1. Association between immunity to hsp and autoimmune disease

Disease	Species	Response described	Reference
A *Mb-hsp60/65 or m-hsp60 vaccination prevents disease*			
Adjuvant arthritis	Lewis rat	– T cell response to Mb-hsp60/65	68
		– A2b specific for Mb-hsp60/65 induces arthritis	67, 68
		– high expression of hsp60 in synovial tissue	37, 14
		– natural antibodies to hsp65 do *not* correlate with AA	22
Streptococcal cell wall-induced arthritis	Lewis rat	– T cell response to Mb-hsp60/65	64
Pristane-induced arthritis	Balb/C mouse	– T cell response to Mb-hsp60/65	26, 62
Diabetes mellitus	NOD mouse	– T cell and antibody response to m-hsp60 and Mb-hsp60/65	17
		– T cell clones (e.g. C9) specific for m-hsp60 induce diabetes	18
B *Mb-hsp60/65 vaccination partially suppresses disease*			
CP-20 691-induced arthritis	Lewis rat		5
Collagen-induced arthritis	Lewis rat		5
	B10RIII mouse	– antibody response to GroEL	28
C *Diseases in which hsp may have a role*			
Rheumatoid arthritis	Human	– T cell response to Mb-hsp60/65	6, 54, 53
		– gamma/delta T cell clones responding to Mb-hsp60/65	26, 61
		– antibodies against Mb-hsp60/65	3, 63
		– expression of hsp60 in synovial tissue	33, 14
		– T lymphocyte-synovial fibroblast interactions induced by mycobacterial proteins in vitro (outgrowth of 'pannus'- like structures)	27
		– only 1 of 26 patients SF T cells proliferated to Mb-hsp60/65	55
		– within a panel of 15 T cell clones obtained from 4 patients 12 different antigenic specificities for mycobacterial antigens were observed	56
Reactive arthritis	Human	– T cell response to Mb-hsp60/65 and h-hsp60	20, 23, 53
		– alpha/beta T cell clone responding to Mb-hsp60/65 and h-hsp60	23
Juvenile arthritis	Human	– T cell responses to Mb-hsp60/65, h-hsp60 and h-hsp70	15
Diabetes mellitus	Human	– antibody response h-hsp60	31
Multiple sclerosis	Human	– colocalization of gamma/delta T cells and hsp65$^+$ oligodendrocytes	58
SLE	Human	– antibody response to h-hsp70 and h-hsp90	44, 43
	MRL (lpr/lpr) mouse	– enhanced expression of hsp70 gene in kidney lymphoid cells	16

induced arthritis in rats[64] and, surprisingly, in pristane-induced arthritis in mice[62] (see table 1). Findings in non-obese diabetic (NOD) mice were even more surprising[17,18]. These mice normally develop spontaneous diabetes by 4 to 6 months of age[13], but it has been shown that immunization with Mb-hsp60/65 in adjuvant at 1 month of age causes an episode of transient diabetes, after which the treated mice do not develop spontaneous diabetes[17]. Thus, Mb-hsp60/65 was shown to induce and subsequently inhibit diabetes.



Peptide epitopes of hsp60 involved in adjuvant arthritis (AA) in Lewis rats and insulin-dependent diabetes mellitus (IDDM) in NOD mice. (for explanations and references see text)

In man, studies have focused on immune recognition of Mb-hsp60/65 in rheumatoid arthritis (RA) and reactive arthritis (ReA) (summarized in table 1). The following key findings support the notion that hsp60 may be involved in the pathology of disease:

1) T cells that recognize Mb-hsp60/65 have been identified in the peripheral blood and in synovial fluid of arthritis patients[6,15,20,23,39,53–56] and they have been used to generate Mb-hsp60/65 specific T cell clones (see the contribution by Kaufmann[35]). The antigen specificity of some of these clones has been studied in detail, e.g. the epitope recognized in vitro by T cell clones derived from the synovial fluid of a patient with reactive arthritis was located within the amino-terminal 15 amino acids[20], whereas the epitope recognized by T cell clones derived from synovial fluid of a HLA-DR4 homozygous patient with rheumatoid arthritis was contained within residues 451 to 471[21] of hsp60 of *Mycobacterium leprae*. However, the in vivo potential of human T cell clones to induce or suppress disease obviously cannot be determined. Consequently, questions remain as to whether these human T cell clones would be pathogenic, or protective, or without effect on the disease process in vivo.

2) A significant enhancement of antibody responses to hsp60 has been noted in patients with RA and ReA (table 1).

3) Using monoclonal antibodies to hsp, restricted local expression of hsp60 was demonstrated in synovial biopsies from RA patients[14, 33, 36], indicating that "self" hsp60 might play a role in the local autoimmune process in the joints of patients with RA. However, it is not clear whether elevated expression of hsp60 in synovial tissues is a cause or a consequence of the disease process[75].

Mb-hsp60/65 and/or endogenous hsp60 have also been implicated in the pathogenesis of other autoimmune diseases in man, such as juvenile arthritis, type I diabetes, and multiple sclerosis (see table 1). Looking at table 1, it appears that immune or autoimmune responses to hsp60 predominate. However, this may well reflect the fact that the majority of studies have concentrated on responses to hsp60 rather than to other hsp. Thus it is noteworthy that in systemic lupus erythematosus (SLE) autoantibodies to hsp70 and hsp90 have been demonstrated[44, 43] (table 1).

Despite the accumulated evidence, the idea that immune responses to hsp (of mycobacteria) are a cause of human autoimmune disease needs to be viewed with some caution. Firstly, mycobacterial hsp which are cloned in *E. coli* might still contain hsp60 (GroEL) from *E. coli*, and the T cell responses observed might be due to reactions to GroEL. Furthermore, because of the striking homology of hsp60 from different bacteria, T cells might respond to cross-reactive epitopes present on hsp of mycobacteria and *E. coli*. Secondly, a number of investigations have been made using antigen preparations such as AP-MT, an acetone precipitate of a water extract of *Mycobacterium tuberculosis*[27]. Although AP-MT contains a high percentage of Mb-hsp60/65, it is possible that some 'impurities' in the preparation may be responsible for the observed effect(s). Thirdly, recent work of Res and colleagues indicates a diversity in mycobacterial antigen recognition by *M. tuberculosis*-specific T cell clones obtained from the synovial fluid of RA patients[56] (see table 1).

On the other hand it appears that T cell responses to Mb-hsp60/65 are not consistently detectable in RA patients. For example, when T cells were obtained from the knee joints of an RA patient, with both knees showing comparable signs of RA, the T cells from only one knee joint responded to Mb-hsp60/65 in vitro[6]. In another patient in the same study, synovial fluid T cells were collected twice within 3 months, and only on one occasion did the T cells respond to Mb-hsp60/65.

Is hsp60 the causative autoantigen?

An ongoing debate is whether the causative antigen in autoimmune disease is hsp as an autoantigen per se, or whether hsp contains epitope(s) cross-reactive (by molecular mimicry) with other autoantigen(s) present in the target organ. Thus, it is of interest that the T cell clone A2b, which induces arthritis in Lewis rats, can be stimulated to proliferate in vitro by extracts of cartilage as well as by Mb-hsp60/65[67, 69]. However, although antibodies against hsp60 stain synovial tissue from the paws of rats with AA[14, 36, 37], the epitope 180–186 of Mb-hsp60/65 which is recognized by the T cell clone A2b is not present in rat-hsp60[66]. Furthermore, although a partial sequence homology of peptide 180–188 of Mb-hsp60/65 with the link protein of cartilage proteoglycan has been described[7, 69], it has been shown that the T cell clone A2b does not proliferate in response to a synthetic nonapeptide corresponding to this sequence of the proteoglycan link protein[66]. Taken together, these results indicate that rat-hsp60 is not the autoantigen recognized by the arthritogenic T cell clone A2b. Therefore, the nature of the autoantigen(s) in the rat joint which are involved in rat AA and cross-react with Mb-hsp60/65 remains to be elucidated.

What is the autoantigen in pristane-induced arthritis in mice (see table 1), where the disease can be prevented by pretreatment with Mb-hsp60/65[62], and how does this observation fit in with the concept that molecular mimicry is involved, since no hsp is contained in the inducing antigen? One possibility is that in pristane-induced arthritis the immune response to hsp may be secondary to the initial inflammatory reaction, which results in increased expression of hsp in the joints involved.

In NOD mice with insulin-dependent diabetes mellitus (IDDM) the cellular and humoral immune responses to m-hsp60 (self) are stronger than those to Mb-hsp60/65 (foreign)[8]. Transient IDDM can be induced, and spontaneous IDDM is prevented, by immunization of NOD mice with hsp60 as mentioned above[17, 18]. This model is the only model so far where purified hsp60 has been shown to induce disease. Thus, it appears that in this model hsp may indeed be the causative autoantigen[8, 19].

In human IDDM, autoantibodies react with a 64 kDa antigen on the surface of beta cells in the pancreas[1]. However, it is highly controversial whether h-hsp60 is[31] or is not[1, 32] the autoantigen. Another candidate autoantigen is the enzyme glutamic acid decarboxylase[2, 59].

With regard to hsp60 as an autoantigen, another critical factor which determines whether an autoimmune response to hsp60 is generated, and whether the response to hsp60 leads to disease, is the genetic background of the animal[60]. In this context, genes within the major histocompatibility complex (MHC) locus play a role in antigen presentation and in shaping the T cell receptor repertoire[4, 46]. Hsp may only mediate an autoimmune disease in individuals with an appropriate MHC background which may, for example, be inefficient in deleting autoreactive T cells and efficient in processing and presenting hsp to those T cells. It is striking that different non-overlapping epitopes distributed over the whole mycobacterial hsp60 are recognized in a MHC-restricted manner, i.e. T cell responses to one highly dominant hsp peptide epitope are supported by different MHC backgrounds[50, 70].

Although it is not formally proven that hsp60 is the causative autoantigen in any autoimmune disease, indirect evidence for this is provided by experiments

where the disease is prevented or modulated by hsp60 (table 1 A, B). It is clear that normal, healthy individuals do have B and T cells specific for hsp[35] without having autoimmune disease. So it is possible that immune responses to hsp are 'normal' and that other influences, e.g. MHC haplotype, the presence of a potential autoantigen, the environment, etc., may lead to disruption of normal immune responses to hsp and thus lead to the development of autoimmune disease (see also the section on Fischer rats in 'Concluding remarks').

From hsp to specific immunotherapy

As mentioned above, T cell clones which can induce autoimmune disease can serve as useful tools to define T cell epitopes in protein antigens, by using peptide fragments of the protein antigen (either synthetic or genetically-engineered peptides), or ideally a series of overlapping peptides that constitute the antigenic part of the protein. The results of such epitope recognition studies for AA in Lewis rats and IDDM in NOD mice[17,18,65,68] are summarized in the figure. To our knowledge, the epitopes involved in the other diseases listed in table 1 have not yet been defined.

Interestingly, all attempts to demonstrate that Mb-hsp60/65 or peptide 180–188 alone can induce AA in rats have been unsuccessful[5,72]. Therefore, we have speculated that induction of AA needs both arthritogenicity and adjuvanticity, which is the case when peptide 180–188 (as Mb-hsp60/65) is present on the surface of mycobacteria[75]. In NOD mice, it has been shown that T cell clones responding to peptide p277 (fig.) can induce diabetes upon transfer to NON.H2^{NOD} mice, which do not develop spontaneous diabetes[8,18]. However, it has been reported that peptide p277 of m-hsp60 is not diabetogenic in NOD mice[8]. This situation is analogous to that with peptide 180–188, which proved not to be arthritogenic in Lewis rats[72]. Thus, it appears that the peptides that stimulate disease-inducing T cell clones in vitro do not induce autoimmune disease in animals.

Are these peptides able to modulate disease? We have addressed this important question in some detail using peptide 180–188 in the Lewis rat AA model[72–74]. Lewis rats can be completely protected against AA by three i.p. injections of peptide 180–188 given 35, 20 and 5 days prior to induction of AA (table 2). We also observed a partial suppression of AA with peptide treatment at the time of arthritogenic challenge with mycobacteria in oil or just before onset of AA[72]. However, a pretreatment regimen with peptide 180–188 which protects Lewis rats against AA does not influence the incidence, onset or severity of type II collagen-induced arthritis in the same rat strain[74]. Therefore we have concluded that peptide vaccination is not only antigen-specific but also disease-specific[19,74,75].

In order to understand the mechanism(s) involved in peptide vaccination, we have examined the immune responses of Lewis rats to peptide 180–188. We found an enhanced cellular immune response to peptide 180–188 and to PPD (purified protein derivative) in the peptide 180–188 pretreated, AA protected rats[73]. The development of IDDM in NOD mice can be prevented by vaccination with p277[8,18,19] (fig.); however, p277 treatment was shown to be accompanied by a decrease in T and B cell immunity to h-hsp60[8,18]. Therefore, the mechanisms of prevention or suppression of autoimmune disease after pretreatment with Mb-hsp60/65 or specific peptides appear to be different in these animal models. Clearly, induction of tolerance[62,64] is not the only mechanism involved in hsp and hsp peptide vaccination[19].

Table 2. Pretreatment with peptide 180–188 protects against adjuvant arthritis (AA)

Exp.[1]	Pretreatment[2] dose mg/rat	Incidence of AA[3]	Radiographical score[4] of individual rats 35 days after induction of AA
1	0	7/7	62, 64, 72, 74, 76, 79, 82
	0.1	3/7	0, 0, 0, 0, 27, 33, 41
2	0	9/9	59, 63, 69, 72, 78, 80, 81, 83, 90
	0.1	2/9	0, 0, 0, 0, 0, 0, 0, 32, 34
3	0	8/8	57, 72, 105, 120, 129, 138, 143, 157
	0.1	15/20	5, 6, 9, 10, 14, 14, 24, 26, 27, 35, 45, 46, 54. 63, 64, 65, 65, 74, 79, 95
4	0	10/10	26, 44, 50, 52, 61, 71, 75, 108, 119, 120
	0.1	8/10	0, 0, 30, 39, 40, 59, 69, 81, 95, 127
	1.0	2/10	0, 0, 0, 0, 0, 0, 0, 0, 1, 6

[1] Experiment 1 and 2 are reprinted from Yang et al.[73] with permission.
[2] Pretreatment was performed on days −35, −20, −5 with peptide 180–188 dissolved in phosphate-buffered saline emulsified in incomplete Freund's adjuvant intraperitoneally.
[3] AA was induced at day 0 by injection of heat-killed, ground *M. tuberculosis* H37Ra intradermally at the base of the tail[73]. Incidence is given according to clinical symptoms such as paw swelling and loss of body weight as a measure of AA.
[4] Radiographs were taken using a Mammomat (Siemens) on Kodak X-omat MA film exposed at 30 kV for 160 ms. 20 joints in each paw were scored visually for arthritic lesions: 0 – normal, 1 – questionable, 2 – mild, 3 – strong, 4 – severe destruction of joints and bone, resulting in a maximum score of 320 per rat[72,73].

Can hsp-peptide vaccination be applied to the treatment of human autoimmune disease?

A prerequisite for the application of hsp-peptide vaccination to human autoimmune disease is the identification of the hsp peptide epitopes involved. In animals it was possible to identify the particular epitopes involved by using T cell clones which had the ability to induce or prevent autoimmune disease in vivo (see fig. 1). The ability of human T cell clones grown in vitro to induce or to prevent disease cannot be tested in man for ethical reasons, particularly in view of the risk of inducing or exacerbating disease. However, attenuated (e.g. irradiated) human T cell clones are already being tested in T cell vaccination in patients with RA or multiple sclerosis to assess the safety and the therapeutic potential of T cell vaccination in man[10].

It should be emphasized that the most successful therapy of autoimmune disease in animal models to date was by treatment with hsp peptide before the induction of AA in Lewis rats, or before the development of IDDM in NOD mice (fig.). Animal models of autoimmune disease, especially adjuvant arthritis, have an explosive onset and rapid progression. Despite this, the T cell clone A2c has been reported to induce earlier remission in the Lewis rat AA model, when A2c cells are injected into the animals just before the peak of disease [25]. Therefore, such therapy may be applicable to man, where the onset and progression of autoimmune disease tend to be relatively slow.

Concluding remarks

Hsp appear to fulfil cellular functions that are fundamental requirements for life, and consequently these molecules have remained highly conserved during evolution. Immune responses to hsp can easily be detected in healthy individuals [29, 35, 41, 48]. In addition, responses to hsp (especially hsp60) are seen in a number of autoimmune diseases, some of which can be prevented by hsp vaccination (table 1). One view may be that hsp are targets for autoimmunity, and autoimmune disease may occur when bacterial infections modulate this natural autoimmunity. However, modulation of hsp autoimmunity by exposure to bacterial hsp can also lead to resistance to autoimmune disease. Thus, it is interesting that Fischer rats, which are class II histocompatible with Lewis rats, are resistant to AA except when kept under germ-free conditions. In other words, exposure of the (mucosal?) immune system to bacterial antigens results in resistance to AA in Fischer rats (reviewed in detail by Van Eden [66]).

If the ubiquitously expressed hsp are immunological targets in autoimmune disease, why are so many autoimmune diseases organ-specific? This may reflect limited expression of autologous hsp in some situations. This view is supported by the observation that hsp60 expression appears to be enhanced locally in the synovial tissue of patients with RA and rats with AA [14, 33, 37]. Alternatively, it could be that other autoantigens, cross-reactive with bacterial or autologous hsp, are the stimulators for the development of autoimmune disease. Support for this view stems from the fact that in many autoimmune diseases immune reactions to other autoantigens have been observed.

It may be possible to develop vaccinations against autoimmune diseases on the basis of hsp or hsp-derived peptides. This has been demonstrated in the Lewis rat AA model, and in NOD mice which develop IDDM (fig.). In addition, therapeutic effects have also been seen in the rat AA model upon treatment with peptide 180–188 [19, 72] (Gasser and Feige, unpublished observations). The partial suppression of ongoing autoimmune reactions in the animal model may have important therapeutic implications for the treatment of human autoimmune disease. Vaccinations using whole hsp proteins might be useful to treat a number of autoimmune diseases, whereas vaccinations based on hsp peptides (i.e. defined T cell epitopes) may lead to disease-specific therapies.

Acknowledgments. We would like to thank T. Hall, R. Tisch, and R. Libleu for critically reading and commenting the manuscript. Also we wish to thank J. Gasser for outstanding technical assistance, B. Riniker for the synthetic peptides, and M. Glatt and I. Wiesenberg for continuous support.

[a] To whom reprint requests should be addressed.

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0014-4754/92/070650-07$1.50 + 0.20/0

Research Articles

Deficiency of fibrinolytic enzyme activities in the serum of patients with Alzheimer-type dementia

T. Aoyagi[a, b], T. Wada[a], F. Kojima[a], M. Nagai[a], S. Harada[a], T. Takeuchi[a], K. Isse[c], M. Ogura[c], M. Hamamoto[c], K. Tanaka[c] and T. Nagao[c]

[a] *Institute of Microbial Chemistry, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141 (Japan),* [b] *Showa College of Pharmaceutical Sciences, 3-chome, Higashitamagawa-gakuen, Machida-shi, Tokyo 194 (Japan), and* [c] *Tokyo Metropolitan Tama Geriatric Hospital, 1-7-1 Aoba-cho, Higashi-Murayama-shi, Tokyo 187 (Japan)*
Received 2 December 1991; accepted 6 March 1992

Abstract. Previously we reported that there is a kallikrein deficiency in the cerebral tissue of patients with Alzheimer-type dementia. The present study was performed to investigate protease changes in the serum of these patients. The results showed that the kallikrein activity was normal, but that the activities of plasmin and urokinase were significantly low. The present findings indicate a derangement in the clotting and fibrinolytic systems in Alzheimer patients.
Key words. Alzheimer's disease; patients' serum; clotting system; fibrinolytic system; plasmin; urokinase; thrombin.

A number of studies have indicated that the accumulation of abnormal proteins in the brain is pathogenetically related to Alzheimer's disease[1–4]. Injection of a protease inhibitor into animal brains was shown to induce the formation of lysosome-associated granular aggregates (dense bodies) which closely resembled the ceroid-lipofuscin that accumulates in certain disease states and in the process of aging[5]. This indicated that abnormal protease activities may play an important role in the development of Alzheimer's disease. In agreement with this hypothesis, we previously found kallikrein deficiency in the cerebral tissues of the patients with Alzheimer-type dementia[6].

In addition to the changes in the cerebral tissues, many biochemical changes have been reported in the peripheral tissues of patients with this disease[7]. They include altered membrane fluidity of platelets[8], increased X-ray sensitivity of fibroblasts[9], reduced free Ca^{++} in fibroblasts[10], reduced fibroblast spreading[11], reduced lymphocyte acetylcholine esterase activity[12], reduced secretion of cholinergic neuron differentiation factor from fibroblasts[13], and decreased adhesiveness of fibroblasts[14].