IMMUNOSUPPRESSION BY RETROVIRUS-RELATED FACTORS
RESTORATION OF p15E-LIKE EFFECTS BY THYMIC HORMONES

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IMMUNOSUPPRESSIE DOOR RETROVIRUS-GERELATEERDE FACTOREN
HERSTEL VAN p15E-ACHTIGE EFFECTEN DOOR THYMUSHORMONEN

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Maarten Pieter Rinse Tas

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PROMOTIECOMMISSIE:

Promotor: prof. dr. R. Benner
Co-promotor: dr. H.A. Drexhage

Overige leden: prof. dr. W. van Ewijk
prof. dr. C.J.M. Melief
dr. R.L. Marquet

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... men moet de mensen zoveel mogelijk leren dat zij niet wijs worden door het lezen van boeken, maar door om te gaan met de hemel en de aarde, met de eiken en de beuken ...

Comenius (17e eeuw)

Aan Paul, mijn vier ouders en Vincent

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VOORWOORD

In een tijd dat AIDS enorm in de belangstelling staat en over de hele wereld onderzoek gedaan wordt naar de eigenschappen van HIV, kom ik met een proefschrift over retrovirale antigenen en immuunsuppressie. Het lijkt alsof wij (dit proefschrift is zeker niet alleen door mij tot stand gekomen), zoals de meeste onderzoekers, de trends heb willen volgen. Het tegendeel is echter waar. Er zijn momenteel maar weinig mensen geïnteresseerd in de werking van retrovirale antigenen. Bovendien betreft het andere onderwerp van mijn proefschrift het herstellend effect van thymushormonen op immuunsuppressie door p15E-achtige factoren. Het is een onderzoek naar de werking van een nog niet compleet gekarakteriseerd medicijn, terwijl tegenwoordig de bekendheid met de structuur van het te gebruiken geneesmiddel nog belangrijker lijkt te zijn dan de werking ervan. Het voordeel van deze goed gekarakteriseerde stoffen is dat de werkingsmechanismen tot in detail uitgezocht kunnen worden. Hemmo Drexhage heeft de afgelopen jaren dan ook z'n uiterste best gedaan om het thymushormoonpreparaat TP-1 waarmee we gewerkt hebben, verder te (laten) karakteriseren. Helaas is dat nooit van de grond gekomen.

Op deze plaats wil ik graag vermelden hoe het mogelijk werd dat ik ooit aan dit promotieonderzoek begonnen ben.

De cito-toets van de lagere school had ik zo slecht gemaakt dat ik niet eens naar de MAVO kon. Mijn moeder was het daar niet mee eens en heeft ervoor gezorgd dat ik het Gymnasium ging doen. Zonder haar was ik waarschijnlijk nooit gaan studeren.

Later ging de invloed van mijn vader werken. Zijn droom was een zoon in de wetenschap. Ik wilde wel medicijnen studeren, maar tot drie keer toe had het lot bepaald dat dat geen studie voor mij was. Medische Biologie bleek een goed alternatief. Het is voornamelijk aan Hanne van Reenen (mijn vaste praktikumpartner) te danken dat ik die studie ooit heb afgemaakt. Vooral in de beginjaren is hij degene geweest die mij bleef stimuleren.

Immunologie, het enige tentamen dat ik ooit over heb moeten doen, werd de richting die ik koos. Hierbij is Rik Scheper van grote invloed geweest. Toen ik mijn kandidaatsexamen biologie wilde aanvragen, bleek dat ik het tentamen Pathologie (dat hoorde bij het Immunologietentamen) nog niet gedaan had. Na het tentamen op het laboratorium waar ik later nog vele monocytpolarisaties zou uitvoeren afgelegd te hebben, vroeg Rik mij of ik het een beetje interessant vond. Dat was zeker het geval. Zodoende heeft Rik voor mij een hoofdvak op de afdeling Pathologie van het RIVM in Bilthoven geregeld.

Een hoofdvak van in eerste instantie een jaar werd er één van officieel 15 maanden. Ik heb daar langer gezeten, vooral omdat ik geen zin had weg te moeten gaan. Het was een stage waar menig student jaloers op kan zijn. Al snel werkte ik samen met de voortreffelijke analiste Liesbeth Geerse. Wij hebben in die tijd enorm veel plezier gehad, maar zijn ook redelijk produktief geweest. Dat konden we opbrengen omdat we de vrijheid hadden om tussen de middag te tafeltennissen (ze won bijna

altijd). De werksfeer was geweldig en alle mensen daar hebben bijgedragen aan mijn interesse voor wetenschappelijk onderzoek: in de eerste plaats natuurlijk Liesbeth, maar niet te vergeten Wim de Jong (die in staat was genoeg aandacht aan mij en vooral Liesbeth te besteden, ondanks dat hij in de laatste fase voor zijn promotie verkeerde), Peter Steerenberg (zijn gesprekken met mij waren als van vader tot zoon), Arja (die verbaasd zou zijn als ik ooit mijn studie af zou maken; dat soort opmerkingen kan soms stimuleren), Jan, Anneke, Anke, Herman, Ingrid en vele anderen.

Het kostte me moeite om op de afdeling Pathologie van de Vrije Universiteit te Amsterdam aan de volgende stage te beginnen. Dat duurde echter niet lang, want ook hier bleek de werksfeer fantastisch. De bijvakken die ik (gedeeltelijk op de afdeling Virologie van de Universiteit van Amsterdam) heb kunnen doen, vallen eigenlijk al onder mijn promotieonderzoek. Dat heb ik te danken aan Hemmo die mij de gelegenheid gaf om bij hem na mijn afstuderen klinische trials uit te voeren die aansloten bij het voorgaande onderzoek, en in de laatste fase ook de afdeling Immunologie van de Erasmus Universiteit te Rotterdam, die mij heeft moeten accepteren als medewerker van Hemmo.

Van het één kwam het ander met tot gevolg dat er nu dit boekje is.

1

INTRODUCTION

1.1 Retroviruses and immunosuppression

Infectious and endogenous retroviruses

Retroviruses are so called because the virus particles contain genes in single-stranded RNA, which can be reversely transcribed into a double-stranded DNA provirus by the enzyme reverse transcriptase of the virus. Retroviruses are classified into three general subfamilies: RNA tumor viruses or oncoviruses (subdivided into A-, B-, C-, and D-type viruses); slow viruses (lentiviruses); and foamy viruses (spumaviruses).

All retroviruses contain at least three genes: gag (nuclear core proteins), pol (reverse transcriptase, protease), and env (envelope glycoproteins). Some retroviruses have additional genes that appear to serve regulatory functions. All retroviruses have a long terminal repeat (LTR) which contains promotor and enhancer regions. An LTR is present at both the 3' and 5' ends of the retroviral RNA, and is duplicated during the viral life cycle.

After a retroviral particle enters a cell, the viral reverse transcriptase makes a double-stranded DNA copy of its genomic RNA. Thereafter the DNA provirus integrates into the chromosomal DNA of the infected host cell. This may remain dormant or be expressed and produce viral proteins and virions (1).

If the infected cell is a germ cell, the integrated provirus will be inherited by all subsequent generations as an endogenous retrovirus. This mechanism of retroviral integration into germ-cell DNA is thought to account for the many classes of endogenous retroviruses present in the germ-line in most, if not all, vertebrate species (2,3). In some species, such as the mouse (4,5) and the baboon (6), some of these endogenous proviruses may be expressed as inducible infectious retroviral particles. In other species, such as African green monkeys or humans, no infectious endogenous retroviruses have been isolated, although their genomes contain abundant retroviral sequences (7,8).

Endogenous retroviral RNA can be reversely transcribed into DNA that then reintegrates back into cellular DNA. This results in multiple copies of the original retrovirus in the genome, which has been described for some classes of endogenous retroviruses (9). Mutations and deletions which have occurred since the initial integration event have rendered almost all endogenous retroviruses non-infectious. These endogenous sequences are considered to be of retroviral origin on the basis of nucleotide homology to the general retroviral structure (LTR-gag-pol-env-LTR). Even in animals not producing intact retroviral particles, the endogenous retroviral DNA may be actively expressed in the form of mRNA and proteins (10-12). It appears that at least 5% of the human and mouse genomes have arisen through reverse transcription of retroviral sequences and transposable elements (13). The striking similarity of retroviral provirus to moveable genetic elements as described for multigene families, such as the immunoglobulin gene family (14) and the interferon gene family (15), has stimulated much interest and speculation, perhaps the most cogent being Temin's hypothesis that retroviruses evolved from transposable elements (16). Over evolutionary time the genome may have been subjected to waves of expansion of particular retroviral-like

elements, as a consequence of an initial germ-line infection and dispersal through transposition or through cellular recombinational mechanisms (5).

Endogenous retroviruses can be expressed in a variety of tissues, such as mammary tissue, epididymis and lymphoid tissue. Lymphocyte endogenous retroviral expression can be enhanced by mitogenic stimulation (17-19). Induction of endogenous retroviral RNA occurs at an early stage of lymphocyte activation since it is detectable within 30 minutes of mitogen stimulation (19). This induction appears to be at least partially due to mitogen-responsive cellular trans-activating factors which bind to the retroviral LTR and regulate retroviral transcription (20).

Infection with animal retroviruses and immunosuppression

The pathological events accompanying retroviral infections are complex. Murine leukemia viruses (MuLV) are the best-studied animal viruses. These viruses are lymphoma-associated. The first isolation by Gross of a leukemogenic virus from a spontaneous murine lymphoma (21) occurred at a time when geneticists had succeeded in identifying several factors or genes that predisposed particular inbred strains of mice to develop a high incidence of spontaneous lymphomas. With the development of sensitive tissue culture and reverse transcriptase assays for MuLV, it became apparent that MuLV could be isolated from most inbred strains of mice, although the great majority of these naturally occurring virus strains were not leukemogenic.

The biological properties of different MuLV isolates and the host resistance towards these isolates are highly polymorphic. It is well established that MHC-restricted T cell responses decisively influence the outcome of virus infection (22). In the rejection of MuLV-induced lymphomas, T helper cells and T cytotoxic cells are considered essential effector cells, besides the effects of MuLV-specific antibodies, which can neutralize the virus and inhibit its spread (23). Recently, it has become clear that failure to express viral antigens (or more rarely MHC class I molecules) at the cell surface of MuLV-induced lymphomas, thereby evading an antiviral immune response, appears to be one of the mechanisms for tumor survival (24). In addition, this mechanism of the virus to escape from immune attack is also of interest for the understanding of tumor survival that animal retroviral infection is accompanied by immunosuppression (25,26).

Dent (25) hypothesized that the impairment of cellular and humoral responses of lymphoid cells following their infection with leukemogenic viruses could be due to disturbances in the local environment affecting the interaction of the immunocompetent cells, to a depletion of these cells, or to a diversion from their normal metabolic and synthetic capacities. However, evidence exists that, apart from the infection of the immunocompetent cells per se with the retrovirus, viral structural components are important in the induction of immunosuppression. In other words, at least some of the immunosuppression caused by retroviruses is the result of the direct effects of produced retroviral immunosuppressive proteins.

In 1977 Olsen et al. (27) and Schaller et al. (28) reported that immunization of cats with UV-inactivated feline leukemia virus (FeLV) abrogated tumor immunity and

increased tumor incidence after challenge with infectious feline sarcoma virus (FeSV), suggesting that retroviral proteins had direct immunosuppressive properties. Similarly, *in vitro* blastogenic responses of feline lymphocytes to concanavalin A (ConA) were suppressed by up to 65% by UV-inactivated FeLV (29). Freeze-thawed preparations of murine Rauscher leukemia virus (R-MuLV) suppressed the *in vitro* blastogenic response of mouse splenic lymphocytes to phytohemagglutinin (PHA) or to allogeneic cells in a two-way mixed leukocyte reaction (MLR) (30), probably due to cell recognition sites being altered by a virion envelope component. In 1978 Mathes et al. (31) reported the inhibition of *in vitro* blastogenic responses of feline lymphocytes to ConA using the purified FeLV envelope protein p15E. p15E is the hydrophobic transmembrane envelope protein (TM-protein) which is synthesized as part of a precursor encoded by the env gene of the murine and feline leukemia retroviruses (32). This precursor is cleaved into p15E and gp70 during protein maturation.

The inhibitory effects of p15E have now been demonstrated for a broad range of cell functions including those of lymphocytes, monocytes and macrophages as summarized in table 1.

In conclusion, the TM-protein p15E of animal retroviruses seems to be one of the important mediators of immune dysfunction associated with animal retroviral infection.

Human retroviruses and immunosuppression

The human foamy virus (HFV) was the first human retrovirus to be described (40). There is evidence that foamy viruses are pathogenic in man and animals. Simian foamy virus (SFV) has caused transient immunosuppression in rabbits (41). Most interesting has been the repeated isolation of foamy viruses from a cluster of patients in Slovakia suffering from de Quervain subacute thyroiditis (42). At that time the agent was not recognized as a foamy virus, although this later became apparent (43).

The second human retrovirus to be discovered, the human T-lymphotropic virus type 1 (HTLV-1), was reported in 1980 (44). HTLV-1 is the etiologic agent of adult T cell leukemia (ATL) (45). The oncogenic potential of HTLV-1 may be reflected in its capacity to transform $CD4^+$ T cells into immortal cell lines (46,47). In tissue culture other cell types can also be infected by the HTLV-1 virus; however, T cells are particularly sensitive to immortalization (48).

HTLV-1 infection has also been associated with immunosuppression since HTLV-1-positive patients with ATL appeared to be unusually susceptible to infection with opportunistic agents, including *Pneumocystis carinii* (49-52), as compared to HTLV-1-negative patients with other forms of T cell malignancies (53). That HTLV-1 may posses immunosuppressive properties is supported by *in vitro* data, showing that HTLV-1 infection of human T cells abrogates their cytotoxic capability (54), their T-helper (54) and delayed hypersensitivity function (55).

Not only viral infection of T cells but also the structural proteins of HTLV-1 themselves may play a role in immunosuppressive mechanisms, as previously described for animal retroviral proteins. Wainberg et al. (56) were the first to report that

Table 1. Immunosuppression associated with the TM-protein p15E.

source of p15E	species of target	function inhibited	refs
FeLV	feline	lymphocyte proliferation	31
FeLV	human	lymphocyte proliferation	33
FeLV	murine	lymphocyte proliferation	34
FeLV	murine	T cell IL-2 secretion	34
FeLV	human	T cell IL-2 secretion	35
F-MuLV[a]	murine	macrophage accumulation	36
R-MuLV	human	monocyte chemotaxis	37
AKR-MuLV[b]	murine	lymphocyte proliferation	38
rp15E[c] (MuLV)	human	lymphocyte proliferation	39

a. Friend murine leukemia virus; b. AKR murine leukemia virus; c. recombinant p15E.

a culture of normal human lymphocytes with either active or UV-inactivated HTLV-1 resulted in suppression of mitogen responsiveness.

In recent years much attention has been devoted to the human immunodeficiency virus type 1 (HIV-1), the etiologic agent of the acquired immunodeficiency syndrome (AIDS) (57). This virus has the capability of selectively infecting and eventually incapacitating the immune system. The critical basis for the immunopathogenesis of HIV infection is a severe depletion of $CD4^+$ lymphocytes, resulting in profound immunosuppression (58-60). There is substantial evidence showing that CD4 is the principal receptor for HIV-1 (61-66). The selective loss of $CD4^+$ lymphocytes occurs in the relatively late stages of HIV infection, namely AIDS related complex (ARC) and AIDS, and is accompanied by functional defects in $CD4^+$ and $CD8^+$ lymphocytes (67). However, immunological abnormalities can also be demonstrated in asymptomatic HIV-seropositive individuals with generally normal numbers of $CD4^+$ and $CD8^+$ lymphocytes. It has become clear that HIV-infection not only affects T cells, but also B cells and monocytes before $CD4^+$ T cell depletion occurs (68). Particularly monocytes, and the monocyte derived macrophages and dendritic cells are infected at an early stage with HIV and are capable of supporting HIV replication (69,70). Monocytes and macrophages may be persistently infected and function as a reservoir for virus dissemination to lungs and brain (69). In symptomatic and asymptomatic HIV-infected individuals HIV infection may induce severe defects in the accessory function of monocytes and peripheral blood dendritic cells (71-73).

By analogy with animal retroviral infection and HTLV-1 infection, it has been

suggested that, besides HIV infection per se of immunocompetent cells, HIV envelope gene products are responsible for T cell loss and that these products may hamper the functions of normal uninfected cells, thereby interfering with the function of the immune system. Fisher et al. (74) suggested a direct role of the TM-protein gp41 in T cell killing by the virus. Sodroski et al. (75) demonstrated that expression of the HIV envelope results in marked syncytium formation depending on the interaction of the HIV envelope and CD4, and concomitant T cell death indistinguishable from the effects of HIV infection itself. Pahwa et al. (76) reported inhibitory effects of disrupted HIV preparations on T lymphocyte blastogenesis, interleukin 2 receptor (IL-2-R) expression, and stimulation of B cell activation. Mann et al. (77) showed that immuno-affinity-purified HIV gp120 binds to the CD4 molecule on the surface of $CD4^+$ helper cells and is able to inhibit the *in vitro* ConA-induced proliferation of normal human lymphocytes.

In conclusion, human retroviruses such as HTLV-1 and HIV modulate immune functions either by a direct infection of cells of the immune system or by mechanisms involving the production of retroviral immunosuppressive proteins. The viral TM-proteins in particular possess immunosuppressive effects.

In chapter 2 of this thesis evidence is presented of a monocyte polarization inhibiting factor in serum of HIV-infected men. This factor, detectable in asymptomatic HIV-infected individuals, ARC and AIDS patients, shares epitopes with the HIV TM-protein gp41, since monoclonal antibodies (mAb) directed against HIV gp41 were able to adsorb the immunosuppressive factor from the serum. In other adsorption experiments, mAb directed against p15E had the same effect as α-gp41, which indicates that gp41 may share epitopes with p15E.

1.2 The amino acid sequence of retroviral transmembrane proteins in relation to their immunosuppressive capability

It has been observed that a 26 amino acid (aa) sequence of the TM-protein p15E is highly conserved in murine, feline, avian, bovine, and simian retroviruses (78-82). To determine whether this highly conserved region in animal retroviruses contained immunosuppressive properties the peptide CKS-17, consisting of the first 17 amino acids of this region in p15E, has been synthesized (83). Indeed, this peptide does have similar effects as p15E and appears to affect both lymphocytes and monocytes in inhibiting several functions as summarized in table 2.

The immunosuppressive CKS-17 amino acid sequence of p15E is also homologous to well-conserved sequences of the TM-proteins gp21 of HTLV-1 and HTLV-2, and, to a lesser extent, gp41 of HIV (38,78,93, see also chapter 2). Synthetic peptides corresponding to the homologous sequences of HTLV-1/2 and HIV to CKS-17 appeared to have similar immunosuppressive properties as CKS-17 itself (94-96), suggesting that the immunosuppression induced by retroviral TM-proteins in general

Table 2. Immunosuppressive properties of the synthetic retroviral envelope peptide CKS-17.

species of target	function inhibited	refs
murine	lymphocyte proliferation	83-86
human	lymphocyte proliferation	83
feline	polyclonal B cell activation	87
human	NK cell activity	88
human	IFN-gamma production	89
human	respiratory burst of monocytes	90
human	monocyte mediated cytotoxicity	91
murine	DTH reactions (*in vivo*)	92

may at least partly be ascribed to the conserved CKS-17 homologues of retroviral TM-proteins. It is worthy to be mentioned that a putative p15E-like TM-protein is encoded by an endogenous C-type human retroviral DNA, containing an amino acid sequence that is partly homologous to CKS-17 (97).

1.3 Endogenous retroviral sequences and immune regulation

It is clear that infection with retroviruses may be accompanied by immunosuppression and that TM-proteins play an important role. As mentioned before, the genomes of most vertebrates contain numerous retroviral sequences, the great majority of which are non-infectious. However, the function of these endogenous retroviruses or sequences is unclear.

Biologic effects of endogenous type C retroviruses

Known biologic effects of endogenous type C retroviral sequences include: participation in leukemogenesis through genetic recombination involving different endogenous viral sequences (98-100); interference with an infection by exogenous retroviruses (101); and the induction of heritable mutations by insertion into the germ line (102-104). Harbers et al. (104), for instance, reported that insertion of the Moloney murine leukemia virus (M-MuLV) into the germ line of Mov-13 mice blocked the transcription of stable alpha 1(I) collagen mRNA and led to a lethal embryonic mutation.

Endogenously produced p15E-like factors in normal mammalian cells

With regard to endogenous sequences related to the TM-protein p15E of animal

retroviruses, peptides from these sequences may be produced by normal cells.

Our group earlier demonstrated the presence of p15E-like material in normal human cells using immunohistological techniques; p15E-like factors were detected in epithelial cells overlaying inflammatory responses of the gut, the skin and the oral mucosa; healthy epithelia were negative (105). In normal conditions positivity was also found in some of the lymphoid tissues, namely in thymus epithelial cells and epithelial cells of the tonsils (105). Using immunofluorescence techniques we were also able to demonstrate p15E-like material in unstimulated monocytes, while only a few lymphocytes were slightly positive (unpublished results). Cianciolo et al. (106) reported that normal human peripheral blood mononuclear cells showed a weak reactivity using mAb directed against p15E. While cultured with ConA, PHA, or pokeweed mitogen (PWM) for 48-72h, these mitogens induced the cells to react stronger with anti-p15E. Moreover, IL-1 has been described to be capable of inducing the production of a retroviral p15E-related chemotaxis inhibitor by endothelial cells (113). However, these factors sharing epitopes with p15E are not necessarily the consequense of the expression of endogenous p15E. Related sequences of p15E-like factors different from TM-proteins may be detected by mAb to p15E.

A role for endogenous p15E-like sequences in immune regulation

Krieg et al. (108) suggested a role for endogenous retroviral p15E-like sequences in the regulation of lymphocyte activation in mice. Antisense oligonucleotides were synthesized complementary to the different initiation sequences of the env genes of three classes of endogenous type C retroviruses, namely ecotropic, xenotropic and Mink cell focus-forming (MCF) env genes. Using these antisense oligonucleotides they were able to determine whether inactivation of these endogenous env products enhanced lymphocyte activation. Although the immunosuppressive CKS-17 sequence is 100% conserved in all three classes of endogenous retroviruses (109-112), only MCF antisense oligonucleotides caused increased spleen cell RNA synthesis and increased cell surface expression of lymphocyte activation markers (108). This stimulation was found to be highly specific for MCF env, since it was only observed with antisense oligonucleotides complementary to the MCF env initiation site. This was not found with control oligonucleotides, or with antisense oligonucleotides directed at other MCF regions, nor with antisense oligonucleotides to xenotropic or ecotropic env initiation sites. That means that other regions beside CKS-17 are important in determining the immunosuppressive effects of the TM-protein p15E, since CKS-17 is highly conserved in all three types of retrovirus.

It is of particular interest that multiple endogenous xenotropic and MCF murine transcripts are induced by polyclonal immune activators, such as lipopolysaccharides and ConA (17-19).

In conclusion, since p15E is immunosuppressive and since endogenous p15E production is inducible from a variety of tissues by immune stimuli, the production of p15E-like factors probably constitutes a physiological circuit that participates in the

regulation of immune homeostasis.

If endogenous p15E-like factors play a role in immune regulation, it is not surprising that these factors are produced as a consequence of an enhanced immune reactivity, and that these factors can be detected in serum. Chapter 3 and chapter 4 of this thesis describe the presence of immunosuppressive p15E-like factors in the serum of patients with chronic purulent infections of the upper airways and in the serum of patients with Graves' disease. The presence of the factor in the serum of these patients was associated with the existence of defects in cellular mediated immunity (CMI), viz., with disturbances in delayed-type hypersensitivity (DTH), monocyte chemotaxis and dendritic cell clustering.

1.4 Aberrant endogenous production of p15E-like factors by malignant epithelial cells

p15E expression by tumor cells

p15E-like factors can also be produced by various tumor cells. It has been shown that these p15E-positive tumor cells are not infected with retrovirus (106). In mice, it has been suggested that the p15E expressed by tumor cells is derived from endogenous retroviral sequences that are present in the germ-line DNA (113). Based on findings on the biologic activity of p15E and the expression of p15E-like factors by a high percentage of murine tumors the hypothesis has been put forward that the expression of these factors supports tumorigenesis by interfering with immunosurveillance (114,115). However, Schmidt & Snyderman (116) reported that in their experiments this expression was neither required nor sufficient for tumor development.

p15E in squamous cell carcinoma of the head and neck

In our group, studies on the presence of p15E-like factors in squamous cell carcinomas of the head and neck have been performed over the past eight years (117,118). The p15E-like factors were prepared after homogenization and centrifugation of tumor material. The supernatants were subjected to ultrafiltration through Amicon CF 25 centriflo cones (molecular weight 'cut off point' 25kD). Using diaflow ultrafiltration and cross-linked dextran gel filtration, we established that the molecular weight of the isolated factors capable of inhibiting the monocyte polarization was between 14 and 16 kD and appeared to be neutralizable by three different mAb to p15E. This indicated that the inhibitory factors present in human head and neck carcinomas shared at least three epitopes with this retroviral component of animal tumor viruses (119). Not only by the way of biologic isolation, but also by use of immunohistochemical techniques, p15E-like molecules were detected in squamous cell carcinoma of the head and neck (105). In these studies all 35 head and neck carcinomas tested for the presence of p15E-like material gave positive results. The p15E-related material was present in the

form of fusiform bands in the cytoplasm of some malignant cells scattered randomly throughout the tumor. These cells were not only found in the superficial areas, but also in deeper layers of the tumor; these deeper layers showed no signs of ulceration. The p15E-like material was not only expressed in head and neck carcinoma cells, but also in six out of ten adenocarcinomas of the breast, five out of eight squamous cell and adenocarcinomas of the bronchus, and nine out of nine adenocarcinomas of the ovarium (105). Regarding these findings it is of interest that Rabson et al. (120) found that, determining human endogenous retroviral DNA sequences that are transcribed into polyadenylated RNA in a number of human tissues, the transcription of human LTR and env sequences was particular prominent in placenta, colon carcinoma and breast carcinoma cells. These authors described that human cells contained a 3.0-kb LTR-env-hybridizing RNA that comigrated with the spliced LTR-env mRNA expressed in MuLV-infected cells, and a shorter, 1.7-kb LTR-env RNA species. Although the sequences of the putative human retroviral env gene did not match the corresponding sequence of MuLV, this region contained a long open reading frame 3' of a potential env mRNA splice acceptor analogous to the structure of murine leukemia viruses. A potential 'gp70-p15E' proteolytic cleavage site could be identified in the human clone which would give rise to a 'p15E' gene region approximately equal in size to that of M-MuLV (120).

Chapters 5 and 7 illustrate the aberrant production of p15E by cancer cells by detecting p15E-like factors in the serum of patients suffering from head and neck squamous cell carcinoma (HNSCC). The relation between the presence of p15E-like factors in serum and the presence of tumor tissue in recurrent or residual malignant disease after treatment is described in chapter 5. In chapter 7 the presence of the p15E-like serum factors is not only related to defects in N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced polarization of patient blood monocytes but also to a defective function of patient blood dendritic cells.

1.5 Evidence that thymic factors restore p15E-like effects

In chapters 3 and 7 the defects in immune functions in patients with chronic purulent rhinosinusitis refractory to antibiotics and surgical treatment, and HNSCC patients could be ascribed to the presence of p15E-like serum factors. Coincidently, we found that thymic hormones may neutralize the effects of p15E-like serum factors in our patient groups. It has been reported that thymic hormones are able to induce raised numbers of circulating T lymphocytes in immunologically impaired patients, as well as improvements in lymphocyte blastogenic responsiveness to mitogens and improvements of DTH skin responses (121-124).

Thymic hormone preparations

Peptides have been isolated from thymic tissue, which have strong effects on the

Table 3. Characterization of the various thymic hormone preparations.

preparation	source	molecular weight	other characteristics	refs
partially purified				
thymostimulin (TP-1)	bovine thymus	1-12 kD		125
thymosin fraction V (TF5)	"	1-15 kD	thymosin-α, γ and ß	126
thymic factor X (TFX)	"	2-18 kD		127
purified				
thymosin-α1	TF5	3100 D	28 aa	128
thymosin-β3	TF5	5500 D		
thymosin-β4	TF5	4980 D	43 aa	129
thymopoietin	bovine and human thymus	5560 D	49 aa	130
thymopentin (TP5)	thymopoietin	550 D	5 aa synthetic	131
thymulin (FTS-Zn)	porcine and human serum	860 D + Zn	9 aa (synthetic)	132
thymic humoral factor (THF)	bovine thymus	< 3000 D		133

functioning of the cell-mediated immune system. During fetal, neonatal and adult life these peptides provide the molecular signals for the generation and maintenance of the thymus dependent immune system. At present, a number of such active preparations, the so-called thymic hormones or thymic peptides, have been prepared from thymic tissue and from blood. These include partially purified preparations, well characterized peptides and synthetic peptides. The substances are listed in table 3.

The effects of thymic hormones on T cells

A major effect of thymic peptides is the induction of differentiation antigens at low molarity on very early precursor T cells in the thymus (134-136). This effect probably represents the physiological signal leading to the appearance of these antigens *in vivo* when pre-T cells reach the thymus. Thymic hormones can also induce or enhance functional maturation in association with other signals of T cell differentiation (137).

Thymic peptides not only play a role in maturation of T cells, but also affect subsets of mature T cells. For instance, several reports exist on the production of lymphokines after stimulation of mature T cells with various thymic hormones (table 4).

Table 4. Main thymic preparations influencing lymphokine production

thymic factor	lymphokines produced	refs
TF5	IL-2, IFN, CSF, MIF	126,138
thymosin-α1	IFN, MIF	128,139
FTS/FTS-Zn	IL-2	132,140
THF	IL-2, IFN, CSF, MIF	141,142
TFX	IL-2, CSF, IFN	127,143
TP-1	IFN, CSF, MIF	125
TP5	IFN, IL-2	144-146

At present it is generally accepted that thymic peptides normalize the immune balance, either by relieving suppressed systems (147-150) or by modulating hyper-responsive states via the stimulation of suppressor mechanisms (140,147). Therefore, it must be assumed that thymic hormones act not only within the thymus, but also at distance, as fine physiological immunoregulators contributing to the maintenance of T cell subset homeostasis. In favor of this hypothesis is the observation of the subtle but significant acceleration of autoimmune diseases and depression of suppressor T cell activity following adult thymectomy (150). However, thymectomy generally has little effect on autoimmune disease unless it is performed in the immediate postnatal period. Smith et al. (151) reported that neonatal thymectomy resulted in the maintenance of the T cell receptor repertoire of early postnatal life, and that this correlated with the subsequent development of organ-specific autoimmune diseases.

The administration of thymic hormones has been described as being effective in several secondary immunodeficiency states, as well as in viral infections and other states of impaired host defence (121-124,146,148-150,152).

The effects of thymic hormones on cells of the mononuclear phagocyte system

Thymic hormones not only exert their effects on T cells, but also on the functioning of cells of the mononuclear phagocyte system. Thymosin-β3 and thymosin-β4 act directly on macrophages to inhibit their migration (153). Tzehoval et al. (154) reported that thymosins modulate antigen presentation of purified murine macrophages and Sztein et al. (155) showed that human accessory cells are an absolute requirement during the late stages of human lymphocyte activation for the full manifestation of thymosin immunomodulating activities (although the interaction of thymosins with T cells did not require the presence of macrophages). Davis et al. (156) reported an effect of TP-1 treatment on serum lysozyme levels in patients with Hodgkin's disease, which suggested an increase in monocyte function and/or mass. There is also a report on a disturbed monocyte natural cytotoxicity in viral hepatitis, the function of which became normal after *in vitro* incubation of the monocytes with thymic preparations (157). Zatz et al. (158) stated that the augmentation of IL-2 production of peripheral blood leukocytes induced by thymosin fraction V was prevented by monocyte

depletion. They interpreted their data as demonstrating that the thymic hormone effect on lymphocyte IL-2 production was mediated directly or indirectly via a monocyte population.

Chapter 6 of this thesis describes the in vivo *restoring effects of TP-1 in a double-blind cross-over trial in patients suffering from chronic purulent rhinosinusitis resistant to the current therapies of surgery and treatment with antibiotics, a defective monocyte polarization, and bioactive p15E-like factors in serum. Beneficial effects on the clinical condition, the purulent nasal discharge and the bacterial invasion of the upper airways are reported as well as a normalization of the depressed fMLP-induced polarization of blood monocytes. The data also clearly show that TP-1 treatment has interferes with the biological effects of p15E-like factors in these patients.*

Chapter 7 of this thesis describes in vitro *studies on the restoring effects of the thymic hormone preparation TP-1 on the defective polarization of monocytes and the defective clustering capability of dendritic cells, isolated from the peripheral blood of patients with head and neck squamous cell carcinoma. Since the defective functions of these cells can be ascribed to the effect of the p15E-like factors produced by head and neck squamous carcinoma cells, the* in vitro *neutralizing capability of TP-1 on the suppression exerted by p15E-like serum factors on monocyte and dendritic cell function was additionally studied.*

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2

A MONOCYTE CHEMOTAXIS INHIBITING FACTOR IN SERUM OF HIV INFECTED MEN SHARES EPITOPES WITH THE HIV TRANSMEMBRANE PROTEIN gp41*

M Tas[1,2], HA Drexhage[1] & J Goudsmit[2]

1. Department of Pathology, Free University Hospital, Amsterdam and
2. Department of Virology, Academic Medical Centre, Amsterdam

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SUMMARY

This report describes that gp41, the transmembranous envelope protein of human immunodeficiency virus (HIV), is able to inhibit monocyte chemotaxis (measured as N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced polarization). To study the presence of such immunosuppressive HIV env proteins in the circulation of HIV-infected men, fractions were prepared from serum via ultrafiltration, yielding molecules with a relative Mr of 25-50. These fractions inhibited fMLP-induced polarization of normal human monocytes, while similar fractions of HIV-uninfected men did not. A monoclonal antibody (mAb) to gp41 was able to adsorb the serum factor responsible for this inhibitory activity. This demonstration of the presence of a gp41-like factor in the circulation of HIV-infected men exerting immunosuppressive activities might have implications for the understanding of the pathogenesis of aquired immunodeficiency syndrome (AIDS), as well as for the selection of HIV-encoded proteins for putative vaccines.

INTRODUCTION

Acquired immune deficiency syndrome (AIDS) is etiologically associated with human immunodeficiency virus (HIV) infection (1,2). Progressive loss of the $CD4^+$ helper/inducer subpopulation of peripheral lymphocytes, accompanied by a diminished T cell performance in delayed type hypersensitivity (DTH) skin tests and blastogenic assays *in vitro* is a hallmark of chronic HIV infection (3), though only a few of the many $CD4^+$ lymphocytes are productively infected by the virus (4). Recently it has been suggested that HIV envelope gene products such as gp41 are responsible for the T cell loss (5-8) and that these products may hamper the functions of normal uninfected lymphoid cells. In this view parallels can be drawn between HIV immunodeficiency and the immunodeficient state occurring in mice and cats after infection with murine and feline leukemia virus (MuLV and FeLV). These latter immunodeficiencies have been ascribed partly to the effect of p15E, the transmembrane protein of MuLV and FeLV (9). p15E suppressed T cell-mediated immune responsiveness as well as having a strong effect on monocyte chemotaxis (10).

Mammalian cells are capable of producing p15E-like factors sharing at least three epitopes with the viral protein of MuLV and with an equal strong immunosuppressive capability (11).

Such production has been described for malignant epithelial cells (12), for lymphoid cells participating in *in vitro* T cell response (13), and for several lymphoid cell lines (11). The p15E-like immunosuppressive factors have also been detected in the serum of patients with immunodeficiencies related to malignancies (12) as well as in HIV-uninfected patients with frequent purulent infections of the skin and upper respiratory tract. It is tempting to speculate that these endogenously produced p15E-like factors are important in (a physiological) regulation of the immune response.

A restricted structural identity (31%) between p15E and HIV gp41 does exist and it has been suggested that p15E and gp41 may therefore share a similar inhibitory function on cell-mediated immunity (14). The region of restricted homology occurs in almost the same region of both molecules.

The present investigation was designed to evaluate the presence of gp41-like and p15E-like monocyte chemotaxis inhibiting factors in the circulation of homosexual men before and after HIV-infection. The inhibitory effects of a HIV-lysate and a HIV gp41, manufactured by recombinant DNA-technique (rDNA gp41), on the chemotactic responsiveness of normal human monocytes was studied as well. Chemotaxis was measured using 'the polarization assay'; this is a rapid and sensitive test system of which the results correlate well with those obtained in the conventional but laborious assay of Boyden (15).

MATERIALS, METHODS AND PATIENTS

Patients

Patients were: (a) five adult homosexual patients with AIDS presenting with opportunistic infections, Kaposi's sarcoma or both; (b) five adult anti-HIV IgG seropositive homosexual patients with AIDS related complex (ARC) exhibiting symptoms as fever, night sweats, lymphadenopathy and an inverted CD4/CD8 ratio; (c) five adult anti-HIV IgG seropositive and (d) five adult seronegative homosexual males without clinical symptoms of AIDS or ARC, who were participating in a prospective study on the prevalence and incidence of HIV infection and risk factors for AIDS in the Amsterdam region; (e) five heterosexual healthy controls.

Serum fractionation

Sera were collected from the patients by venipuncture and diluted 1:1 in saline. These dilutions were subjected to ultrafiltration through Amicon CF50 Centriflo cones (Amicon Corp., Danvers, USA) for 15 min at 700 g (molecular weight 'cut off point' 50kD). The residues were resuspended. The filtrates were again filtered, though Amicon CF 25 Centriflo cones. The residues were resuspended. This yielded three fractions; a fraction of Mr >50, one of Mr between 25 and 50, and one of Mr <25.

Monoclonal antibodies

The following antibodies were used in adsorption experiments: (A) Anti-gp41 (5-10-1, a protein A purified mouse ascites, kindly provided by J. Hunt, Abbott Laboratories, North Chicago, IL, USA); (B) Anti-p15E (4F5-IgG2a, kindly provided by C.J.M. Melief, Central Laboratory of the Blood Transfusion Service, Division Tumor Biology, Amsterdam, The Netherlands); (C) A rat liver araginase, anti-RLA (IgG2a); (D) Anti-HLA-DR (0056B8, kindly provided by L.O. Arthur, National Cancer Institute-Frederick Cancer Research Faculty, Frederick, MD, USA); (E) Anti-p24 (3D3, kindly provided by R. Tedder, Department of Virology, Middlesex Hospital Medical School, London, UK).

Isolation of healthy donor indicator monocytes

Blood samples from healthy donors (aged 20-40 years) were taken by venipuncture and mixed (9:1, v:v) with 3-8% tri-sodium citrate 2-hydrate (Merck, Darmstadt, Germany). The mononuclear leucocyte (MNL) fraction was isolated by Ficoll-Paque density centrifugation (Pharmacia, Diagnostics AC, Uppsala, Sweden). The mononuclear cells were washed three times in Modified Hank's balanced salt solution (Hank's BSS), Ph 7.0, containing 0-38% tri-sodium citrate 2-hydrate and 10 mmol 3-(N-morpholino) propanesulphanic acid (MOPS; Sigma Chemical Company, St Louis, USA) and counted in

a hemocytometer. The percentage of monocytes was determined by a non-specific esterase (NSE) staining and varied from 5-25%. The washed MNL fraction was further treated and layered on top of a discontinuous gradient (Pharmacia, Diagnostics AC, Uppsala, Sweden), with densities of 1.063 and 1.067 and spun at 450 g for 40 min at room temperature. The second MNL fraction thus obtained now contained 60-95% NSE positive cells with a recovery of 40-95% of the number of monocytes originally present.

Polarization assay

We performed a polarization assay according to the method of Cianciolo & Snyderman (10) and added 0.2 ml of the purified MNL suspension, containing 0.2×10^6 NSE positive cells, to 12 × 75 mm polypropylene tubes containing either 0.1 ml Hank's BSS of 0.1 ml of the chemoattractant fMLP, with a final concentration of 10 nM. This fMLP concentration is optimal as has been demonstrated in earlier experiments. All experiments were carried out in duplicate. After 15 min incubation (37°C), the polarization process was stopped by adding 0.5 ml 9% formaldehyde in 0.05 M phosphate-buffered saline solution (pH 7.2). The percentage of polarized cells (for criteria, see below) present in each tube was determined by counting 400 cells in a hemocytometer using an ordinary light microscope (magnification, x250). A cell was classified as polarized when one or more of the following criteria were fulfilled: (1) elongated or triangular shape; (2) broadened lamellipodia; (3) membrane ruffling. The percentage of polarized monocytes was calculated as follows:

$$\frac{\%\ \text{total cells polarized}}{\%\ \text{NSE positive cells}} \times 100\%$$

since it is known that lymphocytes do not polarize in the assay (10).

The effects of HIV-related proteins and serum fractions on monocyte polarization

The effects of a lysate of HIV (3.4 μg/ml in a final dilution of 1:500) and of a gp41 manufactured by rDNA techniques (kindly given by J.A. Hellings, 0.46 μg/ml in a final dilution of 1:500) on the fMLP-induced polarization of the healthy donor indicator monocytes were determined by incubating (15 min, 37°C) the monocytes (1×10^6/ml) either with fMLP alone or with fMLP in combination with these HIV related proteins. The HIV lysate was obtained by lysing the H9 HTLV-IIIB cell line with a mixture of 100 mmol TRIS-HCl pH 6.8 and 1% Triton X-100, followed by heat-treatment (30 min, 50°C). The H9/HTLV-IIIB cell line was kindly provided by R.C. Gallo (Laboratory of Tumor Cell Biology, Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD, USA).

The effects of the serum fractions of Mr of either <25 or between 25 and 50 in final dilutions of 1:60 were also tested in the assay. This to detect low molecular serum factors capable of inhibiting the polarization of healthy monocytes. Polarization was stopped and evaluated as described above. To adsorb the inhibiting factors from these fractions, they were incubated with the different mAb described above (4°C overnight, final dilution of 1:200). HIV lysate and rDNA gp41 were also treated with the mAb. After incubation, the factor-antibody complex as well as unbound mAb was removed via Amicon ultrafiltration. The adsorption procedure was carried out twice.

RESULTS

The effects of a HIV lysate and a recombinant gp41 on fMLP-induced polarization of healthy donor monocytes

The direct effect of the viral env gp41 on monocyte polarization was tested by incubating monocytes of healthy donors with fMLP in combination with either a rDNA

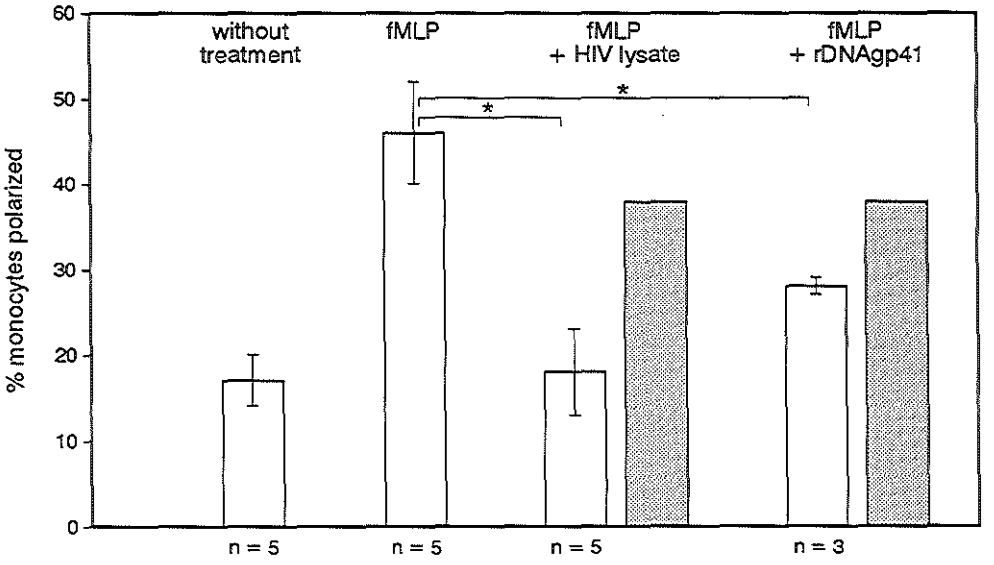

Figure 1. Percent monocytes polarized by treatment with or without fMLP. The ability of HIV lysate (3.4 μg/ml in a final dilution of 1:500) and rDNA gp41 (0.46 μg/ml in a final dilution of 1:500) to inhibit the monocyte polarization was determined by incubating the monocytes with these peptides before adding the fMLP. Results represent means ± s.d. The hatched bars represent experiments after adsorption treatment of HIV lysate or rDNA gp41 with the mAb anti-gp41. * Statistically significant difference ($p<0.05$, Wilcoxon's test) between the values shown.

gp41 preparation or a lysate of HIV grown in H9 cells. In the absence of fMLP, 50-60 healthy indicator monocytes of the 400 cells counted were found to polarize (15-20%, n=5, figure 1); with fMLP added to the system 100-160 monocytes polarized (40-50%, figure 1). By incubating the monocytes with fMLP in combination with the HIV-lysate the effect of fMLP was completely abolished: a value of 18% (mean, s.d. ± 5%) polarized monocytes was found (figure 1). The inhibitory effect of the rDNA gp41 on fMLP-induced monocyte polarization was also evident: a monocyte polarization of only 28% (mean, s.d. ± 1%) was found. The mouse mAb 5-10-1 to gp41 was tested to adsorb the inhibitory effects from the HIV-lysate, as well as from the rDNA gp41; in both instances it was effective to restore the fMLP-induced polarization to values reaching 40% (figure 1). Control experiments were done by incubating the HIV-lysate with mAb to either HLA-DR known to copurify with the virus from H9 cells, or to the viral gag gene product p24. These two mAb were indeed unable to adsorb the monocyte polarization inhibitory factor from the HIV-lysate; the fMLP-induced polarization levels were still at values of 20%, and 25% respectively. Absorption of the HIV-lysate with a mAb to MuLV p15E restored the fMLP-induced polarization to a certain extent, and a value of 35% polarized cells was reached. At earlier occasions (16) we showed this anti-p15E antibody to be able to neutralize almost completely the

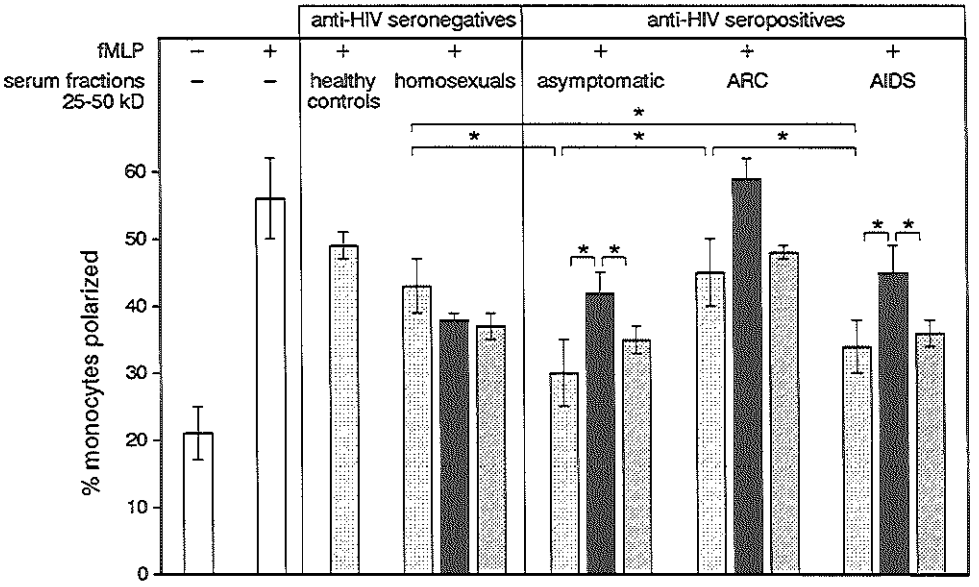

Figure 2. fMLP-induced polarization after preincubating healthy donor indicator monocytes with serum fractions of Mr between 25 and 50 (final dilution 1:60) ▒, the factor was preadsorbed with mAb anti-gp41 ■ and mAb anti-p15E ▒ in separate experiments. Sera were obtained from five adult homosexual AIDS patients, five adult anti-HIV IgG seropositive homosexual patients with ARC, five adult anti-HIV IgG seropositive and five adult seronegative homosexual males without clinical symptoms of AIDS or ARC and five heterosexual healthy controls. Results represent means ± s.d. * Statistically significant difference ($p<0.01$, Wilcoxon's test) between the values shown.

polarization inhibiting effects of endogenous p15E-like factors isolated from head and neck cancers.

The effects of serum fractions of Mr, 25-50 on fMLP-induced monocyte polarization

In this series of experiments (n=7) we found 15-25% of healthy indicator monocytes to polarize in the absence of fMLP, whereas with the chemoattractant present this percentage increased to 55-60% (figure 2). The addition of serum fractions of 25-50kD isolated from the serum of seropositive asymptomatic homosexuals and AIDS patients clearly reduced the fMLP-induced polarization to 30% (mean, s.d. ± 5%) and 34% (mean, s.d. ± 4%) respectively (for statistical significance, see figure 2). Serum fractions obtained from heterosexual healthy controls, seronegative homo-sexuals and ARC patients lacked statistically significant effects on fMLP-induced polarization. In a separate set of experiments it was verified that the serum fractions of the seropositive asymptomatic homosexuals and the AIDS patients had no direct effect on monocyte polarization itself. The effect of the monocyte inhibiting factors of Mr 25-50kD

detectable in the serum in asymptomatic HIV-infected cases and AIDS could be adsorbed with the mAb to gp41: fMLP-induced polarization was restored to 42% (mean, s.d. ± 4%) in the case of the 'AIDS' serum factors (figure 2). The treatment of 'ARC'-serum factors which had in facto no inhibiting effects over those serum factors isolated from normal serum with the mAb to gp41 also had effects: fMLP-induced polarization now reached 59% (mean, s.d. ± 3%) (see figure 2). The mAb to gp41 had no such effects on serum fractions of seronegative homosexuals and healthy controls (figure 2). A control mAb to the rat liver enzyme araginase of the same isotype as the mAb to gp41 (i.e. IgG2) lacked any effects in any of the absorption studies listed above.

The data thus indicate that circulating gp41-like molecules are present after HIV-infections, and that these factors are capable of inhibiting monocyte polarization. The treatment of the positive serum fractions with the mAb to MuLV-p15E had barely any effect (figure 2). It is possible that the affinity of this mAb to the gp41-like factors in serum is too weak to adsorb it.

The effects of serum fractions of Mr <25 on fMLP-induced monocyte polarization

In these series of experiments (n=5), 20-30% of normal indicator monocytes polarized in the absence of fMLP, and 50-70% in the presence of fMLP. Factors <25kD inhibiting fMLP-induced monocyte polarization were only detectable in serum obtained from seronegative homosexuals before HIV-infection (figure 3): fMLP-induced monocyte polarization was significantly lower, and only reached 38% (mean, s.d. ± 3%). In HIV seroconverted cases such factors were no longer detectable (figure 3), and fMLP-induced monocyte polarization values were not statistically significantly different from those obtained in the presence of the serum fraction of healthy heterosexual controls. The monocyte chemotaxis inhibiting factors of Mr <25 present in the HIV-seronegative homosexuals appeared to be antigenically related to p15E, since the inhibitory effect of the serum fractions could clearly be adsorbed with the mAb to MuLV-p15E (figure 3); the mAb to gp41 also had strong effects (figure 3). The control mAb to rat liver araginase lacked any effects.

DISCUSSION

Our studies show that serum fractions of HIV-infected men with an Mr of 25-50 inhibit fMLP-induced polarization of healthy donor monocytes. These *in vitro* immunosuppressive effects could be adsorbed by a mAb to gp41. These data together with the effects shown of an HIV lysate and rDNA gp41 on monocyte chemotaxis can be taken as evidence that viral gp41 or a factor closely related to this env protein circulates in AIDS patients, as well as in asymptomatic homosexual men after HIV-infection. These HIV related nonviable factors in the serum of AIDS patients might be partially responsible for the *in vitro* impairment of monocyte chemotaxis found in such patients (17,18). An inability of peripheral monocytes to migrate to foci of inflammation

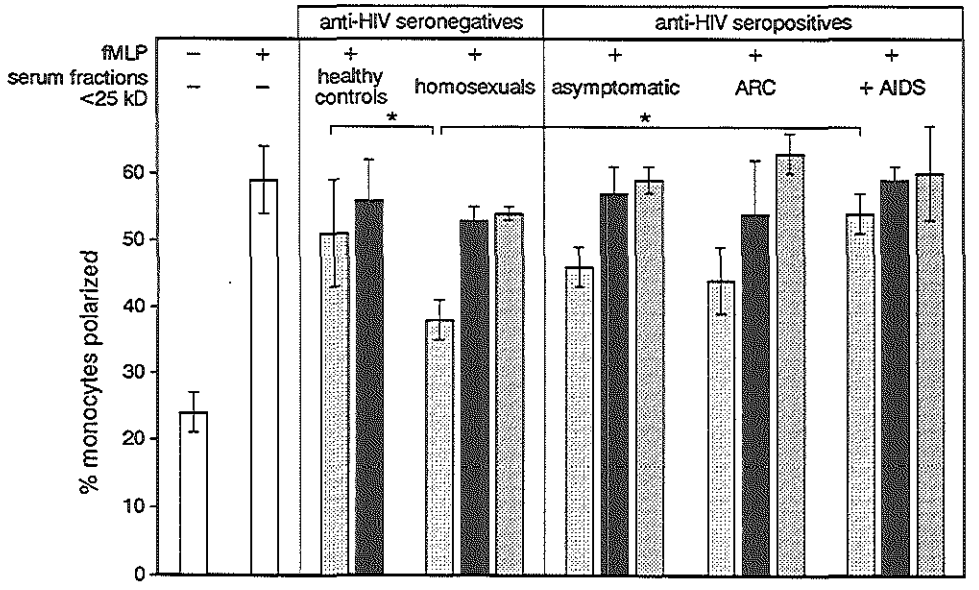

Figure 3. fMLP-induced monocyte polarization by preincubating the indicator cells with serum fractions of Mr <25 or with serum fractions after adsorption treatment with anti-gp41 and anti-p15E . For further details see figure 2.

will certainly contribute to the inadequate host response to opportunistic organisms and neoplasms characteristic of the disease.

In our bioassay the gp41-like serum factor was less active in ARC patients. This difference in serum reactivity between ARC and AIDS is somewhat reminiscent of the reappearance of HIV-Ag measured by ELISA in persons in transition to AIDS (19,20).

The monocyte chemotaxis disturbing properties of HIV gp41 are comparable to those of murine p15E, and our findings that an mAb to p15E was also able to adsorb effects of HIV lysate and gp41 lend additional support to the concept of a restricted homology between p15E and gp41.

Whether the inhibitory effects described by Pahwa et al. (6) of disrupted HIV preparations on T lymphocyte blastogenesis, interleukin 2 receptor (IL-2-R) expression, and stimulation of B cell activation can also be linked to the effect of this sequence needs further clarification. The putative role of nonviable HIV encoded glycoproteins in HIV immunosuppression has been highlighted by others before; Fisher et al. (8) suggested a direct role of gp41 in T cell killing by the virus, and Sodroski et al. (7) demonstrated that expression of the HIV envelope results in marked syncytium formation depending on the interacting of the HIV envelope and the CD4 molecule of T cells, and concomitant cell death indistinguishable from the effects of infection with HIV itself.

Earlier attempts to detect factors in serum responsible for the disturbances in

monocyte chemotaxis seen in AIDS patients have given negative results (17,21). This might be due to the use of unfractionated serum. When unfractionated serum was used in our assay (data not shown) differences in monocyte polarization inhibiting activity between serum from HIV infected and uninfected homosexuals could also not be seen. In fact we found a monocyte chemotaxis inhibitory factor in the serum of homosexuals before HIV-infection but this factor appeared to be smaller than Mr 25. This factor is p15E-like, for the inhibition of the monocyte chemotaxis was reduced to half after adsorption treatment with the mAb to MuLV-p15E. The mAb to gp41 had also this effect.

Similar p15E-like factors have earlier been described in the serum of patients with immunodeficiencies accompanying malignancies (12) and in HIV-uninfected immunodeficient patients with frequent purulent infections of the upper respiratory tract (22). These endogenously produced circulating p15E-like factors are probably important in immune regulations during inflammatory processes and tumorous growth. It is known that homosexuals before HIV seroconversion living in metropolitan surroundings do show defects in cell-mediated immunity and such individuals are frequently affected by chronic infections and sexually transmitted illnesses other than HIV (18).

The homology in function and antigenic structure between HIV gp41 and an endogenously produced immunosuppressive p15E-like factor reported here has analogies in the literature: structural homologies between endogenously produced peptides, playing a role in immune regulation and HIV encoded proteins have now been reported by several groups. Sarin et al. described a cross-reactivity of an antibody to thymosin α_1, with the HIV gag-encoded protein p18 (23). Neuroleukin - a newly discovered nerve growth factor as well as a lymphokine with B cell maturating properties produced by lectin stimulation T cells - shares amino acid sequences with a highly conserved region in the HIV env protein gp120 (24). There is also a region of homology between gp41 and interleukin-2 (IL-2) and this region is said to be associated to the sequences found in p15E and responsible for immunosuppression (25).

These close relationship between HIV-encoded proteins and endogenously produced regulatory peptides raises the question whether an antibody response mounted in AIDS patients to HIV proteins will cross-react with cells known to be involved in the production of these peptides, viz., lymphoid cells and thymic epithelial cells. In this respect it is worth noting that antibodies to lymphoid cells have been reported in ARC and AIDS patients (26). Moreover, a thymic dysplasia has been described in AIDS showing large numbers of plasma cells and the loss of epithelial architecture, morphological signs suggestive for autoimmune destruction (27-29).

The notions listed above highlight the caution that should be made in selecting HIV antigens for vaccination: these might by themselves be immunosuppressive or cytopathic to immune cells. Moreover, the antibody response raised to such antigens could be damaging to the host's regulator mechanisms of cell-mediated immunity.

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3

ABNORMAL MONOCYTE CHEMOTAXIS IN PATIENTS WITH CHRONIC PURULENT RHINOSINUSITIS: AN EFFECT OF RETROVIRAL p15E-RELATED FACTORS IN SERUM*

EM van de Plassche-Boers, M Tas, M de Haan-Meulman, M Kleingeld & HA Drexhage

Laboratory for Clinical Immunology, Pathological Institute, Free University, Amsterdam

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SUMMARY

Earlier we reported that about 60% of patients suffering from unexplained relapsing of chronic purulent rhinosinusitis show a defective T cell-mediated immunity to commensal micro-organisms of the upper respiratory tract. The monocyte chemotactic responsiveness was assessed in 40 of these patients by means of the polarization assay. Impaired N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced monocyte polarization was found in 26 of the 40 patients tested. The defective chemotactic responsiveness could be explained by a p15E-related factor detectable in the serum of the patients: addition of serum fractions <25kD to healthy donor monocytes resulted in an inhibition of polarization; a monoclonal antibody (mAb) directed against p15E neutralized this inhibitory effect. In individual patients, a decreased monocyte polarization correlated well with the presence of this p15E-related factor in serum, as well as with defective T cell reactivity.

INTRODUCTION

A disorder frequently encountered in ENT and pediatric practice, is a relapsing of chronic purulent rhinosinusitis, in spite of several courses of antibiotics and surgical intervention to improve drainage of the sinuses. As reported earlier in detail, 60% of these patients show a defective T cell-mediated immunity to commensal micro-organisms of the upper respiratory tract (1). The T cell impairment was detected using delayed type hypersensitivity (DTH) skin testing and the macrophage migration inhibition factor (MIF) production by peripheral lymphocytes; both tests showed a faulty reaction to somatic antigens prepared from *Haemophilus influenzae,* to *Candida albicans* and to streptococci (1-4). Other T cell functions, viz., the blastogenic responsiveness towards these antigens and the number of peripheral T cells, were normal (4), indicating that the microbe-specific T cell functions were only partially defective.

An impaired function of monocytes often accompanies T cell abnormalities; this has been well documented in atopic dermatitis (5-7), in the human immunodeficiency virus (HIV)-caused syndromes LAS and AIDS (8,9) and in immunodeficiencies accompanying various types of malignancies (10-14).

The presence in serum of factors capable of inhibiting the function of both lymphocytes and monocytes has been reported, particularly in malignancies. Factors of Mr <25kD capable of inhibiting IL-2 production and monocyte chemotactic responsiveness were detected in serum, urine and cancerous effusions of cats (15,16), mice (17) and men (14,18-20). These factors appeared to share structural homology with the feline and murine retroviral transmembrane protein p15E (18) as could be shown by adsorption studies using mAb against this immunosuppressive viral protein.

The hydrophobic transmembrane protein p15E is highly conserved among many type C and type D retroviruses. A structural homology between p15E and transmembrane components of other retroviruses has recently been described (21). A number

of human cell-lines derived from lymphocytic and monocytic neoplasms as well as normal phytohemagglutinin (PHA)-stimulated lymphoblasts were found to produce p15E-like factors (22). Apparently p15E-like factors can be endogenously produced by lymphocytes, monocytes and squamous epithelial cells, moreover the factors could also be detected by immunohistochemical methods in epithelial cells overlaying areas of inflammation (23), and in normal thymic epithelial cells (unpublished observations). This suggests that the p15E-like factors play a role in normal immune regulation.

In this paper we report on defects in chemotactic responsiveness of monocytes in the above-mentioned patients with a relapsing of chronic purulent rhinosinusitis. The presence of p15E-related factors in serum, capable of depressing monocyte chemotactic responsiveness was established as well. The chemotactic responsiveness was determined using the 'polarization' assay; the polarization of human monocytes towards chemoattractants is an early event that precedes their chemotactic responsiveness (24). The conventional 'Boyden chamber' assay has some disadvantages such as laborious and time-consuming test procedures and the requirement of special equipment and a relatively large blood sample. These disadvantages are overcome by the 'polarization' assay (24); a modification of this test developed in our laboratory was used (14). The assay correlates well with the Boyden chamber method (14,24).

MATERIALS AND METHODS

Patients

40 patients, 16 males and 24 females, aged 20 - 60 years (median 42 years) with a relapsing of unexplained purulent rhinosinusitis were studied. Criteria for inclusion in the study were (1,2):

a. a positive culture for *H. influenzae, S. pneumoniae,* other streptococci, or staphylococci at one or more occasions;
b. no response to or only temporary relief after treatment with several courses of antibiotics;
c. failure of surgery to improve the drainage of the ethmoidal and maxillary sinuses to give a permanent cure;
d. no detectable gross disturbances in mucociliary transport (exclusion of Kartagener and related syndromes; 25);
e. duration of the disease of at least 18 months;
f. normal levels of total serum IgG, IgM and IgA;
g. total numbers of peripheral blood leukocytes and differential white cell count within normal limits;
h. normal or slightly raised *H. influenzae*-specific IgG and IgM antibody titres (which are detectable in all healthy individuals tested; 3,4);
i. no treatment with antibiotics and/or other drugs known to influence the immune system, at the time of testing.

Healthy controls

Twenty-five healthy hospital staff members, 12 males and 13 females, aged 22 - 40 years (median 31 years), with a negative personal and family history of respiratory disorders, atopy and autoimmunity, volunteered as controls.

Parameters of T cell functions

DTH skin test reactivity towards three commensal microbial antigens, a somatic antigen derived from *H. influenzae*, candidin and Streptokinase/Streptodornase (SKSD), was measured as described previously (3). MIF-production of peripheral lymphocytes upon stimulation with these three antigens was estimated as described in detail elsewhere (4).

Isolation of mononuclear leukocytes

Blood samples were obtained by venipuncture and mixed (9:1, v:v) with tri-sodium citrate 2-hydrate. The mononuclear leukocyte (monocyte) fraction was isolated by Ficoll Paque density gradient centrifugation. After isolation, the cells were washed three times in phosphate buffered saline (PBS), pH 7.4, containing 0.38% tri-sodium citrate 2-hydrate and 0.5% bovine serum albumin (BSA) and counted in a hemocytometer. The number of monocytes was determined in suspension employing positive staining with non-specific esterase (NSE) (26). The percentage of NSE-positive cells varied from 5-25%. An enrichment for the monocytes in the Ficoll Paque isolated fraction was obtained by Percoll gradient centrifugation (27). After washing, the pellet containing the monocytes was resuspended in Medium 199 (pH 7.0 and containing 0.03% L-glutamine, 1% BSA and 0.084% NaHCO3) and carefully underlayed with equal volumes of Percoll 1.063 and 1.067. After centrifugation (40 min 450 g) the cells were collected from the interface, washed twice in Medium 199 (10 min 500 g) and counted: the suspension now contained 40-60% NSE-positive cells.

Polarization assay

The Cianciolo and Snyderman assay (14) was performed with slight modifications (24). Aliquots (0.2 ml) of the Percoll purified cell suspension containing 0.2 x 10^6 monocytes were added to 12-75 mm polypropylene tubes (Falcon Labware Division of Becton Dickinson Co., Oxford, CA, USA) containing 0.05 ml of either Medium 199 or N-formyl-methionyl-leucyl-phenylalanine (fMLP) in Medium 199, to reach a final concentration of 10 nM. All experiments were carried out in duplicate. The tubes were incubated at 37°C in a waterbath for 15 min. The incubation was stopped by addition of 0.25 ml ice-cold 10% formaldehyde in 0.05% PBS (pH 7.2). The cell suspensions were kept at 4°C until counting in a hemocytometer using an ordinary light microscope (magnification 250x). The test was read 'blindly' by two persons; 200 cells were counted from each tube. A cell was 'polarized' if any of the following occurred:

1. elongated or triangular shape
2. broadened lamellipodia
3. membrane ruffling.

The percentage of polarized monocytes was calculated as follows:

$$\frac{\%\ \text{total cells polarized}}{\%\ \text{NSE-positive cells}} \times 100\%$$

Lymphocytes do not exhibit any polarization activity towards fMLP (14). A good correlation has been found in our laboratory between the chemotactic responsiveness towards casein in the Boyden chamber assay and the percentage of polarized monocytes when using fMLP; it is suggested that this validates the polarization assay (14).

Serum fractionation

Sera were collected from the patients by venipuncture and diluted 1:1 in saline. These dilutions were subjected to ultrafiltration through Amicon CF25 centriflo cones (Amicon Corp., Danvers, USA) for 15 min at 700 g (molecular weight 'cut off point' 25kD). The residues were resuspended and stored at -70°C until further use.

Adsorption experiments

Adsorption experiments were carried out with serum fractions by incubation with mAb in a final dilution of 1:200 at 4°C for 16 hours, followed by Amicon ultrafiltration to remove formed complexes. The whole procedure was repeated. The mAb used were anti-p15E (4F5-IgG2a, kindly provided by Dr. C.J.M. Melief, Central Laboratory of the Blood Transfusion Service, Division Tumor Biology, Amsterdam, The Netherlands) and a mAb with the same isotype, but a specificity for an unrelated antigen: anti rat liver araginase (anti-RLA, IgG2a).

Inhibition of polarization

The ability of the serum fractions to inhibit fMLP-induced polarization of healthy donor monocytes was determined by incubating the monocytes (1×10^6/ml) for 15 min at 37°C either with fMLP alone or with fMLP in combination with a serum fraction (final dilution 1:60). Addition of serum fractions alone to donor monocytes did not effect the polarization. The polarization test was performed as described above, and the percentage of inhibition of fMLP-induced polarization minus spontaneous polarization caused by addition of the serum fractions calculated.

RESULTS

T cell functions

In the group of 40 patients reported here, antigen-specific T cell defects towards somatic antigen from *H. influenzae*, to candidin and to SKSD could again be detected in about 60% of cases. Twenty-four of the 40 patients showed a defective DTH skin test as well as a faulty MIF-production. The outcomes of both assays correlated extremely well, and in 38 patients $\chi^2 = 31.9$; $p<0.001$. Fifteen patients were defective in both assays towards one of the antigens, eight patients towards two of the antigens and one patient towards all three antigens. Two exceptions were encountered. In one patient normal skin tests to all three antigens were accompanied by faulty MIF-production towards two of the antigens (*H. influenzae* and SKSD). In another patient a defective skin test was found towards candidin, and a faulty MIF-production was recorded towards *H. influenzae*.

Monocyte polarization

Total numbers of peripheral blood monocytes were within the normal range in all patients ($5 - 80 \times 10^4$ monocytes/ml blood, mean 30×10^4). For details see Balm et al. (13).

Figure 1 shows the results of the polarization assay in 40 patients and 25 healthy controls. The percentage spontaneous polarization was within similar ranges for both patients and controls (7-20%). This is in agreement with results reported earlier by Tan et al. (14) (controls: 13.5 ± 6.5%; patients 11.9 ± 5.4%). However, the fMLP-induced polarization was decreased in 26 of the 40 patients, when compared to the values found in healthy controls: patients 28.1 ± 8.8%; controls 38.7 ± 7.3%; fMLP-induced minus spontaneous polarization: in controls 25.2 ± 4.6%, in patients 16.1 ± 9.3%.

A good correlation was found between a decreased monocyte polarization assay

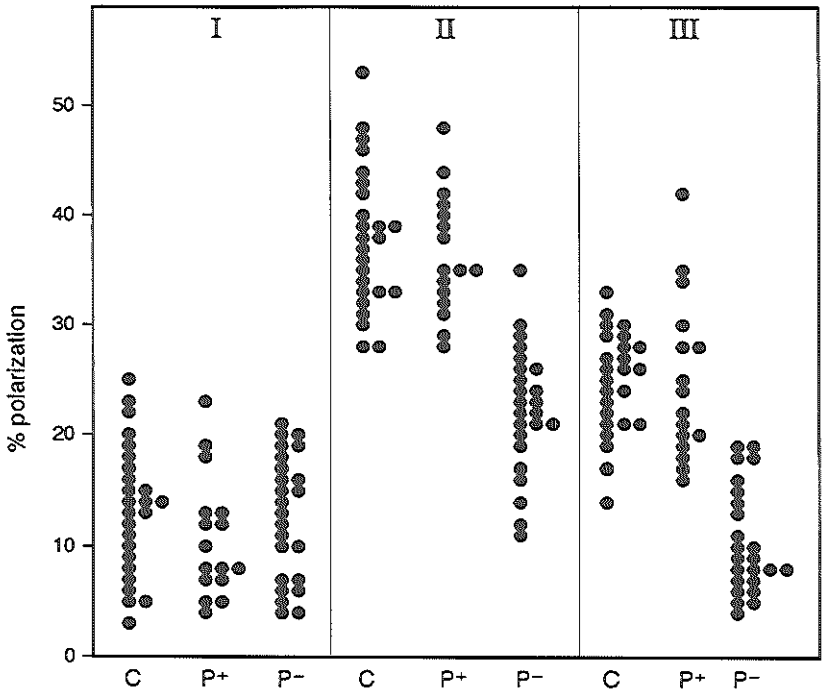

Figure 1. Monocyte polarization assay. I, unstimulated (spontaneous) polarization; II, fMLP-stimulated polarization; III, II - I. C, healthy controls (n = 25); P+, patients with normal T cell functions (n = 16); P—, patients with decreased T cell functions (n = 24).

and the presence of defective DTH skin test and MIF test results. In 37 of the 40 patients the results of monocyte function and T cell parameters were concordant (χ^2 = 27.8, $p<0.001$). In two patients with normal values of monocyte polarization, DTH skin tests and MIF-production were defective. In one patient the T cell parameters were normal, but the polarization values were decreased.

Low molecular weight inhibitory factors

Sera from 24 patients and 12 controls were ultrafiltrated on Amicon filters to yield fractions of Mr <25kD, which were tested in four dilutions on healthy donor monocytes for the presence of inhibitory factors. In the serum fractions from healthy individuals, some inhibitory effect was detectable, but dose-response relationships could not be established (table 1). The level of inhibition varied between individuals, up to a maximum of 35% (inter-individual variation), when tested on the same donor monocytes. Testing of the same serum fractions on monocytes from different donors also revealed fluctuations in the level of inhibition (intra-individual variation), but values never exceeded the inter-individual variation of 35%. Values of inhibition <35% were thus considered

Table 1. Effect of serum fractions <25kD on the polarization of healthy donor monocytes.

	% Inhibition of polarization			
dilution of serum fraction	1:3*	1:6	1:12	1:24
healthy individuals n = 12	17.2 ± 10.4	16.6 ± 12.2	19.9 ± 8.6	17.2 ± 9.4
patients A n = 7	20.0 ± 10.7	23.6 ± 9.6	18.0 ± 11.8	19.3 ± 11.1
patients B n = 17	49.7 ± 17.7	41.7 ± 12.8	35.4 ± 17.5	17.5 ± 11.2

* Dilution of serum fraction. Final dilutions in assay: 20 x higher. For each concentration mean % of inhibition of polarization ± s.d. is given.
Patients A: have normal values of % polarization of their monocytes.
Patients B: 14 of the 17 patients have a decreased % polarization of their own monocytes.

to be insignificant in further experiments.

The effect of the serum fractions from seven patients was entirely comparable to healthy controls: inhibition did not exceed the 35% level. The serum fractions from 17 patients showed higher levels of inhibition of fMLP-stimulated polarization of healthy donor monocytes and dose-response effects were clearly detectable (table 1). This indicates that factors <25kD capable of inhibiting monocyte polarization were present in their sera. Since in nearly all patients a 1:3 dilution of the fractions resulted in the highest percentage of inhibition, this dilution was used in further experiments.

Of the 17 patients possessing low molecular weight inhibitory factors, 14 belonged to the group whose own monocytes showed a decreased polarization and three patients had a normal polarization test. The seven patients without low molecular weight inhibitory factors all had a normal polarization test. Consequently, a good correlation was found in individual patients between the presence in serum of high levels of inhibitory factors <25kD and a disturbed chemotactic responsiveness of circulating monocytes (χ^2 = 13.8, $p<0.01$).

Adsorption of serum fractions with anti-p15E

The serum fractions <25kD from three healthy individuals and five patients with decreased monocyte polarization were adsorbed with four dilutions of a mAb directed against p15E, as well as with a mAb directed against an unrelated antigen, rat liver araginase (RLA). In controls, effects from anti-p15E treatments were not detectable, but in patients this treatment had clear neutralizing effects in all cases. A representative example of the results obtained in the patients is shown in figure 2. In all five patients tested, the anti-p15E treatment of the serum fractions resulted in a dose-dependent

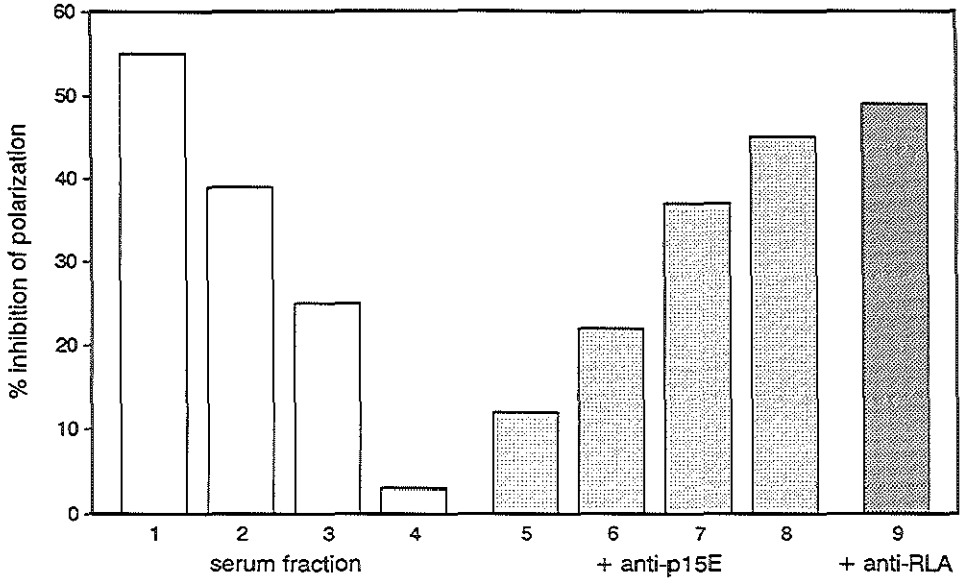

Figure 2. Effect of adsorption on the polarization of healthy donor monocytes. All results shown were obtained by simultaneous testing of serum fractions from one patient: 1 to 4 shows the effect of different dilutions of serum fractions <25kD on donor monocyte polarization. Serum dilutions used: 1=1:3 (final dilution in assay 1:60), 2=1:6, 3=1:12, 4=1:24. In 5 to 8 the effect of adsorption of serum fraction 1 (=dilution 1:3) with different dilutions of anti-p15E is shown. Dilution of anti-p15E 5=1:3, 6=1:6, 7=1:12, 8=1:24, 9 shows the lack of effect of adsorption of serum fraction 1 with monoclonal antibody of the same isotype, but specificity for the unrelated antigen rat liver araginase.

decrease of the inhibiting effect, whereas anti-RLA had no effect at all. Since in practically all patients the optimal effect was seen when using anti-p15E in a 1:3 dilution, a larger group of patients and controls was tested using this concentration of anti-p15E.

Figure 3 shows the effects of such anti-p15E treatment in 12 controls and 24 patients. Serum fractions <25kD which had insignificant effects on monocyte polarization (<35% inhibition), were not affected by the anti-p15E treatment. These serum fractions were obtained from the healthy individuals and from three patients with normal T cell functions and monocyte polarization.

The serum fractions <25kD obtained from 21 patients which had significant inhibitory effects on donor monocyte polarization could clearly be neutralized by anti-p15E in all cases. All of these patients had a decreased polarization of their own monocytes and T cell defects.

DISCUSSION

Earlier we reported data on DTH and MIF defects in patients suffering from a relapsing of purulent rhinosinusitis refractory to antibiotics and surgical treatment (1-4). In this paper we report that the chemotactic responsiveness of monocytes, as measured by the polarization assay, is also defective in about 60% of these patients. A concordance between the presence of decreased monocyte chemotaxis and a defective T cell function was established.

A possible cause for the defective immune responsiveness was found in the presence of inhibitory factors in the patients' serum, which appeared to be p15E-related. Such increased levels of serum factors inhibiting monocyte chemotaxis have been well documented in patients suffering from thermal injuries (29), several types of tumors (18,20,22,30-34) and have also been detected in sera of HIV-seronegative homosexuals with a high risk for AIDS (35).

The origin of the p15E-related factors in chronic purulent rhinosinusitis is speculative. The putative possibilities range from exogenous infection with an as yet unknown retrovirus possessing envelope substances that share structural homology with p15E (it is worthy to be mentioned that several of our patients clearly indicate a

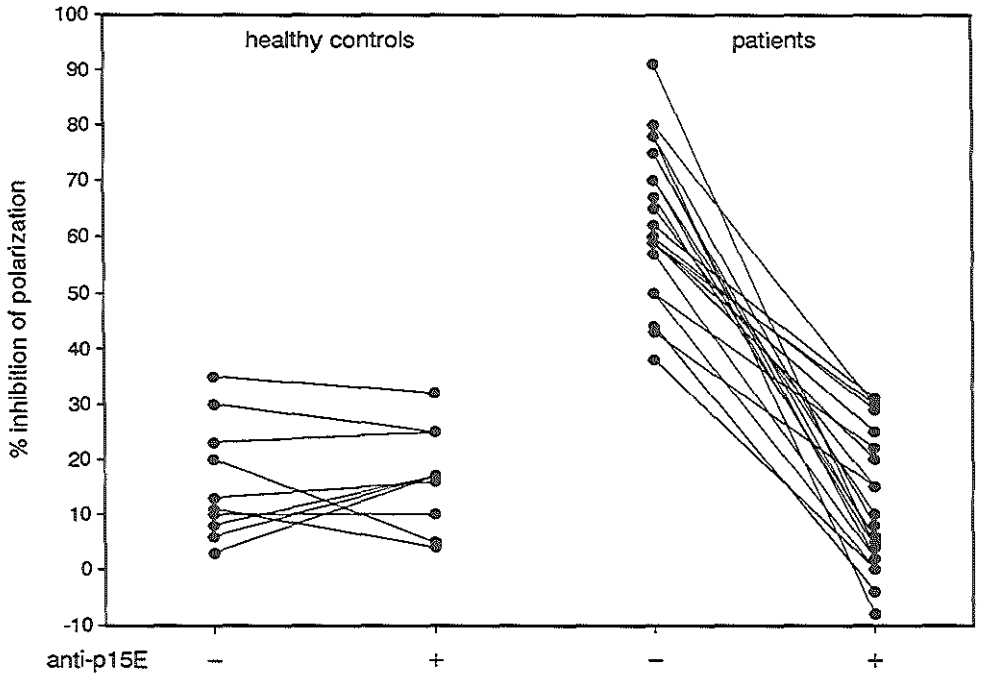

Figure 3. The percentage inhibition of healthy donor monocyte polarization caused by serum fractions <25kD before and after adsorption with anti-p15E. Serum fractions (1:3) were adsorbed with anti-p15E (1:3). Controls: n=12. Patients: n=21 with disturbed polarization of their own monocytes.

virus infection just prior to the onset of their chronic upper respiratory tract complaints), to an endogenous production of the factors; it is known that p15E-related factors can be produced by lymphocytes, monocytes, thymic and mucosal epithelial cells (23,36).

The mechanisms by which p15E inhibits fMLP-induced monocyte polarization are also speculative, but interference of p15E with receptors influencing motility seems likely. Specific receptors for the N-formylated peptides have been detected on the surface of human monocytes (19,37,38). Monocytes that do respond to fMLP are also capable of responding to other N-formylated oligopeptides, as well as to other chemoattractants such as zymosan-activated human serum (AHS) and lymphocyte-derived chemotactic factor (LDCF). Apparently, responsive monocytes share receptors for different chemotactic stimuli. This view is supported by the findings that the p15E-like factors in human serum are capable of inhibiting the polarization induced by fMLP as well as by AHS and LDCF (24). On the other hand, it has been reported that on neutrophils, oligopeptide receptors appear to be different from receptors for other chemoattractants (38). In this respect it is worthy to note that the fMLP-induced neutrophil chemotactic responsiveness is disturbed independently of that of the monocytes in our patients (unpublished observations) and that this has also been reported in tumor patients (18).

p15E-like factors are also known to influence the function of T cells. For instance, they have been reported to inhibit IL-2 production of normal T cells *in vitro* (39) and the factor is produced in mitogen-driven lymphocytic transformation assays (22). It might be possible that the factors are also capable of affecting other T cell functions. Our observation of a good correlation between decreased DTH skin tests/MIF production and the presence of p15E-related factors in serum supports such a view. Another possibility might be that the p15E-disturbed monocyte functions have consequences for the functioning of the T cells (via antigen presentation, IL-1 production etc.). CKS-17, a synthetic peptide which corresponds to part of the highly conserved retroviral p15E region of homology, was reported to inhibit IL-2-dependent lymphocyte proliferation (40). We are now investigating the effect of this peptide on the fMLP-induced polarization of monocytes.

In conclusion, our results clearly indicate a relationship between the presence of p15E-like factors in serum and a disturbed immune function in patients suffering from a therapy refractory form of chronic purulent rhinosinusitis. In a pilot study, thymic hormone treatment was given to improve the immune responsiveness of the patients. Preliminary results indicate an effectiveness: a disappearance of p15E-like factors from the serum was found concomitant with clinical improvement. A controlled clinical trial involving a larger group of patients is presently carried out.

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4

DEFECTS IN MONOCYTE POLARIZATION AND DENDRITIC CELL CLUSTERING IN PATIENTS WITH GRAVES' DISEASE

A PUTATIVE ROLE FOR A NON-SPECIFIC IMMUNOREGULATORY FACTOR RELATED TO RETROVIRAL p15E*

M Tas[1], M de Haan-Meulman[1], PJ Kabel[2] & HA Drexhage[1]

1. Department of Immunology, Erasmus University, Rotterdam and
2. Department of Clinical Immunology, Academical Hospital, Utrecht

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SUMMARY

A depressed chemotactic responsiveness of monocytes and a depressed clustering capability of dendritic cells have been found in diseases such as chronic purulent infections of the respiratory tract and in various types of malignancies. These impairments in monocyte and dendritic cell function could be ascribed to the action of a low molecular weight factor (LMWF; <25kD) circulating in the serum of the patients. The factor, which seems to be a non-specific immunoregulatory factor, shares a structural homology with p15E, the capsular protein of murine and feline leukemogenic retroviruses.

In order to study the chemotactic responsiveness of monocytes and the clustering capability of dendritic cells of Graves' patients, monocytes were isolated from the peripheral blood and dendritic cells were prepared from these peripheral blood monocytes by exposure to metrizamide. Monocytes were studied for their chemotactic responsiveness measuring their capability to polarize (morphological changes determined by light microscopy) after stimulation with the chemoattractant N-formyl-methionyl-leucyl-phenylalanine (fMLP). Dendritic cells were studied for their capability to form clusters with allogeneic lymphocytes. A defective fMLP-induced monocyte polarization was found (16 vs 37% in healthy controls), whereas the dendritic cells showed a defective clustering (60 clusters vs 151 clusters in healthy controls). The effect of fractions of <25kD prepared from the serum of Graves' patients on healthy donor monocytes and dendritic cells was studied to test the presence of p15E-like factors. The serum fractions had a significant inhibitory effect on monocyte polarization and dendritic cell clustering. A mixture of two monoclonal antibodies directed against p15E was able to neutralize these suppressive effects. The relationship of this p15E-like factor occurring in the serum of Graves' patients to the recently reported genomic expression of retroviral sequences in thyrocytes/leukocytes of Obese Strain chickens and Graves' patients (ev 22, HIV gag) needs further clarification.

INTRODUCTION

In Graves' goiters the numbers of intrathyroidal monocytes and of macrophages and dendritic cells (both monocyte-derived) are higher as compared to the numbers found in normal thyroid glands. Monocytes are found around the capillaries; macrophages are often seen infiltrating the colloid and engaged in colloidophagy; dendritic cells are often seen in contact with intrathyroidally infiltrated lymphocytes. The raised numbers of monocytes, macrophages and dendritic cells and the intimate contact of the latter cells with lymphocytes is highly suggestive of an active involvement of monocytes and monocyte-derived cells in the autoimmune process of Graves' disease (1,2).

Macrophages normally have a scavenger function, whereas dendritic cells are the antigen presenting cells and of crucial importance in the presentation of antigen

during the elicitation of primary and secondary immune responses. The most characteristic feature of the dendritic cells in comparison to other class II positive cells is their capability to actively form clusters with immunocompetent cells in their vicinity and to create a microenvironment suitable for optimal antigen presentation and T and B cell activation (3).

This report firstly describes an investigation on the chemotactic responsiveness of monocytes isolated from the blood of Graves' patients using the so called 'polarization assay'. The polarization assay is a rapid and easy method to measure monocyte chemotactic responsiveness and is based on the changes in shape of monocytes ('polarization') under the influence of the chemoattractant N-formyl-methionyl-leucyl-phenylalanine (fMLP). These changes are considered to precede monocyte chemotaxis (4).

Secondly, the capability of dendritic cells from the peripheral blood monocytes of Graves' patients to form clusters with allogeneic lymphocytes was studied. Early clustering of dendritic cells with lymphocytes is considered to be antigen-independent and the first step in accessory function and antigen presentation (5). The process is dependent on adhesion molecules (6,7) but not on the expression of MHC class II determinants (7). *In vitro* clustering formation of dendritic cells with lymphocytes results in the production of lymphokines. The development of responsiveness of the lymphocytes to the lymphokines, Ig synthesis and the initiation of the mixed leukocyte reaction (MLR) also occurs in the clusters (5,8). Consequently, it has been found that the number of dendritic cells and the number of formed clusters in mixed leukocyte reactions correlated well with lymphocyte stimulation (9).

Defects in monocyte polarization and the clustering of dendritic cells prepared from these monocytes have been found in diseases such as chronic purulent rhinosinusitis (10,11) and in immunodeficiencies accompanying various types of malignancies (12-14). In these disorders the presence in serum of a low molecular weight factor (LMWF; <25kD) capable of suppressing monocyte polarization and dendritic cell clustering is well documented (10,12,15).

We therefore extended our studies and investigated the putative presence of this retroviral p15E-like factor in the serum of patients with Graves' disease and its influence on the polarization of healthy donor monocytes and the clustering of dendritic cells prepared from such monocytes.

PATIENTS AND METHODS

Patients and controls

The following groups were studied:

a. Heparinized blood was obtained from Graves' patients visiting the Internal Medicine or Surgery department. Monocytes and/or dendritic cells were isolated. On first presentation these patients had hyperthyroidism, raised serum T4 and T3 levels, decreased serum thyroid stimulating hormone (TSH) or absent TSH responses to thyrotropin-releasing hormone (TRH) and a diffuse non-nodular appearance of the thyroid on palpation, scan or ultrasonography (n=11; eight females, three males; ages ranging from 22 to 45 yrs). Two patients had clear

Graves' eye signs. Practically all (n=9) were treated for their hyperthyroidism with carbimazole and had normal T3 and T4 values at the time of testing. Two patients were new admissions and had active Graves' disease with raised serum T4 and T3 and low serum TSH.

b. Heparinized blood was also obtained from patients visiting the Endocrine Clinic for Graves' ophthalmopathy problems (n=7; five females, two males; ages ranging from 33-63 yrs). Five were treated with carbimazole, two had active eye disease. Serum was available from these seven patients for the determination of p15E-like factors. The thyrotropin binding inhibiting immunoglobulin (TBII; TRAK-assay, Henning, Berlin, FRG) values were determined in six patients; two were in the normal range whereas three positive (values over 10% inhibition).

c. Serum for the determination of p15E-like factors was further obtained from a serum bank of TBII+ Graves' patients (n=10; eight females, two males; ages ranging from 14 to 63 yrs). All these patients were newly diagnosed cases and hence had not been treated. TBII ranged from 10.6 to 72.7%.

d. Controls were healthy laboratory personnel (n=44; ages ranging from 22 to 43 yrs). Both serum and heparinized blood was collected from these subjects.

The isolation of peripheral blood monocytes

Peripheral blood mononuclear cells from patients and healthy controls were isolated by Ficoll-Isopaque density gradient centrifugation. The cells were washed twice in phosphate buffered saline (PBS), pH 7.4 containing 0.5% bovine serum albumin (BSA), and counted in suspension employing positive staining with non-specific esterase (NSE) (16). The percentage of NSE-positive cells varied from 5 to 25%. An enrichment for the monocytes in the Ficoll-Isopaque isolated fraction was obtained by Percoll gradient centrifugation (17): after washing, the Ficoll-isolated pellet containing both monocytes and lymphocytes was resuspended in RPMI 1640 supplemented with 10% fetal calf serum (GIBCO, Breda, The Netherlands) and carefully underlayed with an equal volume of Percoll 1.063 (Pharmacia, Diagnostics AC, Uppsala, Sweden). After centrifugation (40 min, 450 g) the cells were collected from the interface, washed twice in medium (10 min, 500 g) and counted: the suspension now contained 70-95% NSE-positive cells. This suspension was directly used for the monocyte polarization and maturation of monocytes to obtain dendritic cells.

From three different healthy donors monocytes were isolated by counterflow elutriation centrifugation. These almost pure monocyte fractions were used to test the effects of the LMWF. In brief, mononuclear cells were separated from 450 ml whole blood of the healthy controls via Percoll centrifugation (20 min, 1000 g, room temperature). Thereafter, the mononuclear cells were injected into an elutriation centrifugation system (Beckman J21 centrifuge with a JE-6 elutriation rotor). The elutriation medium was PBS with 13 mM trisodium citrate and 5 mg of human albumin per ml. To separate the different cell populations, the flow rate was kept constant at 20 ml/min, while the rotor speed decreased from 4000 to 0 rpm. The fraction collected at 2500 rpm contained 93-97% monocytes as judged by positivity for NSE activity. This fraction was used in further experiments after storage in liquid nitrogen.

Metrizamide treatment of monocytes to obtain dendritic cells

Dendritic cells were prepared from peripheral blood monocytes according to the method described by Kabel et al. (9). Metrizamide (Serva, Heidelberg, FRG) was dissolved in RPMI supplemented with 10 % fetal calf serum. The isolated monocytic fractions were exposed to metrizamide in suspension culture for 30 min. Thereafter, the cells were washed (culture fluid was added slowly to prevent osmotic lysis of the cells), and further cultured under non-adhering conditions for 16 h in polypropylene tubes (5% CO_2 and 37°C, 100% humidity). This procedure yields 40-80% cells with a dendritic morphology, showing class II MHC positivity, a decreased expression of the monocytic CD14 determinant, a decreased phagocytic capability, but an enhanced stimulator capability in the MLR. The full technical details of this method are given in Kabel et al. (9).

The monocyte polarization assay

The Cianciolo and Snyderman (4) assay for monocyte polarization was performed with slight modifications (14). The assay has proved to be a rapid method for testing monocyte chemotaxis and outcomes of the assay correlate well with outcomes of the conventional Boyden chamber assay to measure chemotaxis to casein (14). 0.2 ml aliquots of the Percoll or elutriator purified cell suspension containing 0.2 x 10^6 monocytes were added to 12-75 mm polypropylene tubes (Falcon Labware Division of Becton Dickinson Co., Oxford, CA, USA) containing 0.05 ml of either medium or the chemoattractant fMLP in medium, to reach a final concentration of 10 nM. All experiments were carried out in duplicate. The tubes were incubated at 37°C in a waterbath for 15 min. The incubation was stopped by addition of 0.25 ml ice-cold 10% formaldehyde in 0.05% PBS (pH 7.2). The cell suspensions were kept at 4°C until counting in a hemocytometer using an ordinary light microscope (magnification, ×250). The test was read 'blindly' by two persons; 200 cells were counted from each tube. A cell was 'polarized' if any of the following occurred:

1. elongated or triangular shape
2. broadened lamellipodia
3. membrane ruffling

The chemotactic responsiveness of a monocyte population was expressed as the percentage of polarized monocytes in the presence of fMLP minus the percentage of polarized monocytes in the absence of fMLP. The percentage of polarized monocytes was calculated as follows:

$$\frac{\%\text{ total cells polarized}}{\%\text{ NSE-positive cells}} \times 100\%$$

Lymphocytes do not exhibit any polarization activity in this assay (4). Of 77 healthy control individuals tested during the last two years a mean of 33% polarized monocytes was found (s.d. 11%; range 18-70%). There were no differences between female and male individuals: females, a mean of 33%, s.d. 9% (n=36); males, a mean of 32%, s.d. 13 (n=41). Nor were differences found between individuals less than 50 years and over 50 years of age: respectively, a mean of 34%, s.d. 11 (n=66); and a mean of 31%, s.d. 8 (n=11). The inter-assay never exceeded 17% (n=13); the intra-assay variation never exceeded 15% (n=77). On the basis of these outcomes fMLP-induced polarization values of less than 20% polarized monocytes are considered to be abnormal.

Clustering of dendritic cells

The cluster assay as described by Austyn et al. (3) was performed with modifications (9). $5x10^4$ dendritic cells prepared from peripheral blood monocytes exposed to metrizamide were allowed to cluster with $5x10^3$ allogeneic lymphocytes isolated from healthy controls (4 hours, 37°C, 5% CO_2) in 250 μl flat-bottomed wells. The lymphocyte isolation was performed according to standard procedures with Ficoll-Isopaque and nylon wool adherence (Leuko-Pak, Fenwall Laboratories, IL, USA). Formed clusters were counted using an inverted microscope and values were expressed as the number of clusters per six microscopic fields (x200). A cluster was defined as an accumulation of 4-25 cells.

The determination in patient serum of LMWF inhibiting monocyte polarization and clustering of dendritic cells

Sera were collected from the patients by venipuncture and diluted 1:1 in saline. These dilutions were subjected to ultrafiltration through Amicon CF25 Centriflo cones (Amicon Corp., Danvers, USA) for 15 min at 700 g (molecular weight 'cut-off point' 25kD). The residues were resuspended and stored at -70°C until further use.

The capability of the serum fractions to inhibit fMLP-induced polarization of healthy donor monocytes (elutriator purified) was determined by incubating the monocytes ($1x10^6$/ml) for 15 min at

37°C, either with fMLP alone or with fMLP in combination with a serum fraction (final dilution 1:60). The percentage of inhibition was calculated as follows:

$$i = (1 - (Ff\text{-}fo/Mf\text{-}fo)) \times 100\%$$

Ff = % monocytes polarized after incubation with fMLP and LMWF
Mf = % monocytes polarized after incubation with fMLP alone
fo = % spontaneously polarized monocytes

Addition of serum fractions to non-fMLP stimulated donor monocytes did not affect the spontaneous polarization.

The capability of LMWF to inhibit the clustering of dendritic cells was determined by incubating dendritic cells ($5x10^4$) with allogeneic lymphocytes ($5x10^3$) and with or without the LMWF (final dilution 1:6; 4 hours, 37°C 5% CO_2).
The percentage of inhibition (i) was calculated as follows:

$$i = (1 - F/M) \times 100\%$$

F = number of clusters in presence of LMWF
M = number of clusters in presence of medium

The serum fractions were tested in triplicate.

Adsorption experiments of the LMWF with monoclonal antibodies to p15E

To validate the p15E-like character of the LMWF, adsorption experiments were carried out by neutralizing the serum fractions before testing in the monocyte polarization and dendritic cell clustering with a combination of two p15E specific monoclonal antibodies (see below) in a final dilution of 1:200 at 4°C for 16 h, followed by Amicon ultrafiltration to remove formed complexes; this adsorption/neutralizing procedure was carried out twice (18). The monoclonal antibodies used were a combination of 4F5 and 19F8 (anti-p15E isotypes IgG2a and IgG2b; kindly provided by Dr. G.J. Cianciolo, Genentech Inc., Pharmacological Sciences, South San Francisco, CA, USA). As a control antibody we used anti-human IgG (Tago, Burlingame, CA, USA).

RESULTS

The fMLP-induced polarization of monocytes isolated from the blood of patients with Graves' disease

Figure 1 shows the results of fMLP-induced monocyte polarization of 12 patients with Graves' disease as compared to the outcomes of 28 healthy control subjects tested in this period. In patients with Graves' disease a clearly diminished percentage of monocytes were polarizing under the influence of the chemoattractant fMLP (mean 16%, s.d. 9 vs 37%, s.d. 8; $p<0.01$). Although the series is limited it is noteworthy that the three patients with newly diagnosed, untreated, active Graves' disease had values of 12, 12 and 7% respectively and therefore were not statistically different from the carbimazole treated patients. The six cases of Graves' ophthalmopathy also did not statistically differ from the other Graves' disease cases.

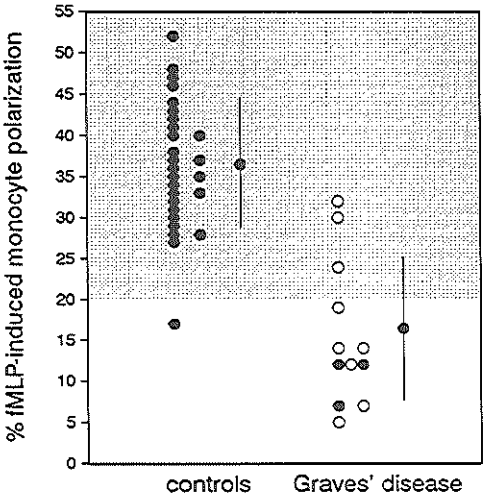

Figure 1. FMLP-induced monocyte polarization. A clearly diminished percentage of polarized monocytes after exposure to fMLP was found in the Graves' disease patients (Wilcoxon, $p<0.01$). Patients treated with carbimazole (○); untreated patients (●). The hatched area represents normal values.

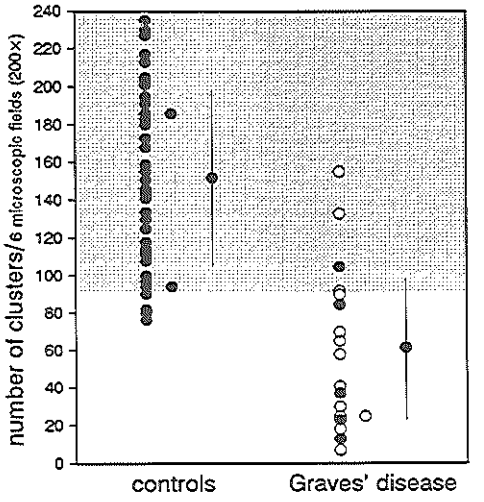

Figure 2. Number of clusters of dendritic cells with allogenic lymphocytes (six microscopic fields; 200x). The capability of blood dendritic cells from Graves' patients to form clusters with allogeneic lymphocytes was disturbed compared to healthy controls (Wilcoxon, $p<0.01$). Patients treated with carbimazole (○); untreated patients (●). The hatched area represents normal values.

Clustering of dendritic cells with allogeneic lymphocytes

The recovery of dendritic cells from the monocytes of patients and that of healthy controls was in the same range (40-60%). When the Graves' dendritic cells were allowed to cluster with allogeneic lymphocytes a difference from healthy controls became apparent. The clustering capability of Graves patients' dendritic cells was lower than that of healthy controls; namely, a mean of 60 clusters/six microscopic fields (x200), s.d. 43, $n=18$, was found in Graves' disease, whereas a mean of 151 clusters was found in healthy controls (s.d. 48, $n=36$; $p<0.01$; figure 2). Again, there was neither a difference between the five active, untreated Graves' patients vs the carbimazole-treated patients, nor between the cases with or without Graves' ophthalmopathy.

Figure 3 shows a comparison of the data obtained in the monocyte polarization assay with those data obtained from the same individuals in the cluster assay. Numbers are too small to calculate a reliable correlation coefficient. It can be seen, however, that the lower the monocyte polarization, the lower the dendritic cell clustering. This indicates that the disturbances in Graves' monocytes and monocyte-derived Graves' dendritic cells are related.

p15E-like factors

Since the defects in fMLP-induced monocyte polarization and dendritic cell clustering might be explained by the presence of a p15E-like LMWF with immunosuppressive capabilities in the serum of the patients (see introduction), the activity of this factor in the serum of 11 untreated Graves' patients, 10 of whom were TBII+, as well as in five carbimazole treated patients was tested. Figure 4 shows the effects of serum fractions of <25kD on the polarization of healthy donor monocytes (figure 4a) and on the clustering capability of healthy donor dendritic cells with allogenic lymphocytes (figure 4b). The LMWF of the Graves' patients clearly inhibited fMLP-induced monocyte polarization (mean 50% inhibition, s.d. 12 vs 14% inhibition by healthy control LMWF, s.d. 6, $p<0.01$; Wilcoxon). The LMWF also had an inhibitory effect on the clustering capability of healthy donor dendritic cells, though this inhibition was less marked (mean 28% inhibition, s.d. 11 vs 16% inhibition, s.d. 6). The inhibitory effect of the LMWF on both cell populations was neutralizable with a mixture of two monoclonal antibodies directed against p15E: the inhibition exerted by the LMWF was alleviated after adsorption of the serum fractions with the anti-p15E monoclonal antibodies and values of 16%, s.d. 8 (monocyte polarization) and 13%, s.d. 5 (dendritic cell clustering) were found (figure 4). The control monoclonal antibody (an anti-IgG) had no neutralizing effect (data not shown). This supports the p15E-like character of the Graves' LMWF. Figure 5 shows the comparison of the presence of the p15E-like LMWF in the serum of the tested individuals with the actual monocyte polarization (figure 5a) and dendritic cell clustering (figure 5b). The numbers are too small to calculate reliable correlation coefficients, but it can be seen that the higher the p15E-like factor in the serum (patients), the lower the monocyte polarization or dendritic cell clustering.

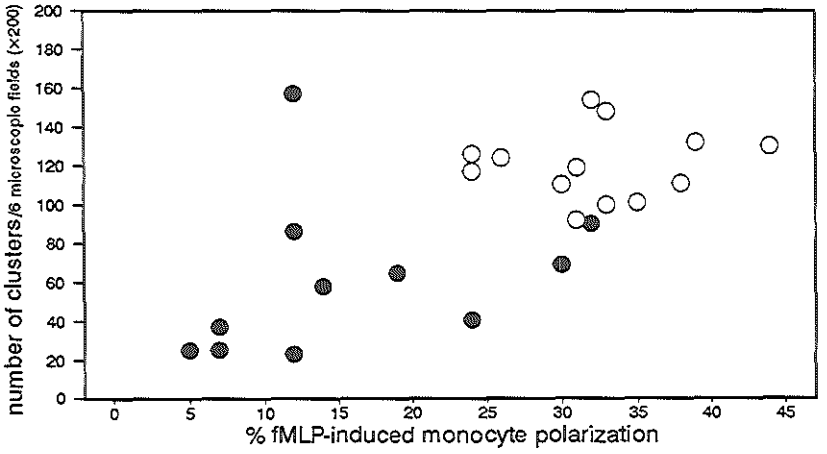

Figure 3. The comparison of the data obtained in fMLP-induced monocyte polarization with those obtained in dendritic cell clustering. Patients (●); healthy controls (○).

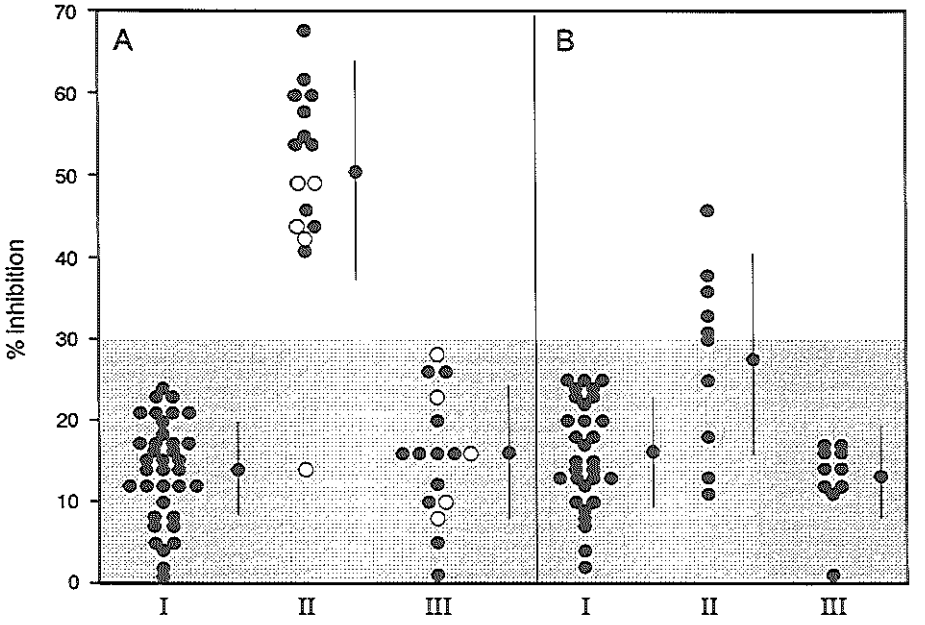

Figure 4. The effects of serum fractions <25kD on a, the polarization of healthy donor monocytes and b, on the clustering capability of healthy dendritic cells with allogeneic lymphocytes. I, Healthy control serum fractions; II, Graves' serum fractions and III, Graves' serum fractions after adsorption with anti-p15E. The hatched area represents normal values. Patients treated with carbimazole (○); untreated patients (●). The hatched area represents normal values.

DISCUSSION

The hydrophobic transmembraneous protein p15E is highly conserved among many type C and type D retroviruses and the protein is responsible for the immunodeficiencies seen after infections with these retroviruses (19). Endogenous immunosuppressive factors of <25kD which share a structural homology with p15E do also occur in non-infected organisms and such factors were firstly described in the serum, the urine and the cancerous effusions of cats (20), mice (21) and men (12, 13, 19). These endogenous factors are capable of inhibiting monocyte polarization, dendritic cell clustering, IL-2 production and NK-cell activity (10, 12, 15). The p15E-like factors have also been found in the serum of type I diabetes patients (unpublished observations) and most notably in the serum of patients with purulent airway infections and clinical signs of defects in cell mediated immunity (10, 22).

A number of human cell lines derived from lymphocytic and monocytic neoplasms as well as normal PHA stimulated lymphoblasts are capable of producing the p15E-like factors. Retroviral infection of these cells and cell lines was excluded since neither complete viruses nor other components of the viruses were

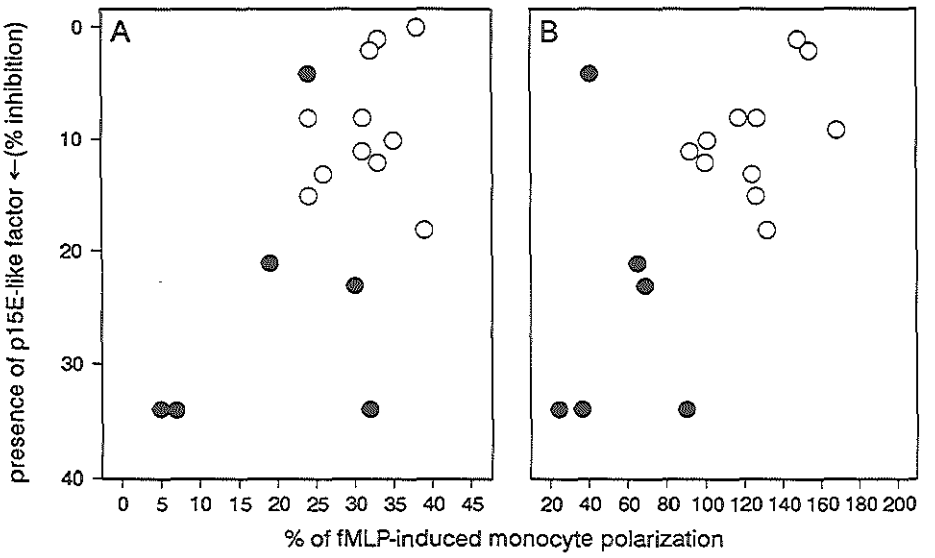

Figure 5. The comparison of the presence of the p15E-like factors, expressed as inhibition of the serum fraction <25kD minus the inhibition of the serum fraction after adsorption with anti-p15E, with a. the monocyte polarization and b. dendritic cell clustering. Patients (●); healthy controls (○).

present. p15E-like factors can also be detected by immunohistochemical methods in epithelial cells overlaying areas of inflammation (18), and in normal thymic epithelial cells, monocytes and spleen macrophages (unpublished observations). Apparently, the p15E-like factors can be produced by a variety of mammalian cells, including lymphoid cells and epithelial cells. We believe that the endogenously produced p15E-like factors play a role in immune regulation in general and may be considered as cytokine-like factors. Structural homologies between immunoregulatory cytokines and retroviral components have been described before (23,24).

This study shows the presence of p15E-like factors in the serum of Graves' patients. These patients were characterized by a defective chemotactic responsiveness of blood monocytes (as measured in fMLP-induced polarization) and a defective clustering of blood dendritic cells with allogeneic lymphocytes. Since clustering is based on the presence of adhesion molecules (see introduction), it is of interest that recently a lower expression of the adhesion molecule LFA-1 has been found on peripheral blood lymphoid cells from patients with Graves' disease (25).

Our data cannot be explained by the immunosuppressive effect of carbimazole treatment on monocytes and monocyte-derived cells as found by Weetman et al. (26,27). Firstly, in the carbimazole treated and untreated Graves' patients from our series similar defects in monocyte polarization and dendritic cell clustering were found. Secondly, in the 10 untreated TBII+ patients p15E-like LMWF were detecta-

ble. The presence of the immunosuppressive factor in the serum and the defects of blood monocytes and blood monocyte-derived dendritic cells in the Graves' patients are in contrast to the seemingly high activity of the monocytes and dendritic cells in the Graves' thyroid gland itself, namely an enhanced infiltration of monocytes and dendritic cells and intimate contacts of intrathyroidal dendritic cells with lymphocytes have been observed (1,2). The presence of the p15E-like factor in serum might be explained by assuming that the factor is produced in response to an intrathyroidal enhanced (auto)immune response, thus representing an immune counterregulatory mechanism. The factor can also be detected in the serum of patients with other thyroid autoimmune conditions, such as Hashimoto goiter and thyroid atrophy (a limited series of three patients was tested). Apparently the effect of the serum factor is not strong enough to result in clinically overt cell-mediated immunodeficiency, since such overt deficiencies have never been reported in thyroid autoimmunity. It must be noted that in our group of patients with chronic purulent airway infections with clinically overt defects in cell-mediated immunity higher levels of serum p15E were found (55% inhibition of monocyte polarization, s.d. 15, (10), versus 34% inhibition due to p15E-like factors from Graves' patients, s.d. 16, this report).

The origin of the p15E-related factors in Graves' disease is speculative. Apart from the above mentioned epithelial cells and lymphoid cells, we have found that normal thyrocytes contain p15E-like material in a fine granular pattern using immunohistochemical techniques; Graves' thyrocytes however contained less or no p15E-like material. Recently the expression of other retroviral sequences was reported in thyroids affected by autoimmune disease, namely of the Obese Strain chicken (28) and of Graves' patients (29).

Chickens of the Obese Strain develop a hereditary spontaneous autoimmune thyroiditis which closely resembles human Hashimoto's disease. Analysis of the genome of these animals revealed a new endogenous avian leukosis virus sequence (ev 22), which was suggested to have a modulatory role in the development of the disease. Ciampolillo et al. (29) found a positive hybridization signal on Southern blot analysis of Graves' thyroid gland and lymphocyte DNA with probes for gag HIV. Their findings were explained as suggesting the presence of a novel retrovirus in Graves' disease and the retrovirus-like sequences seemed to be closely associated with thyroid autoimmunity.

The relationship between the p15E-like factor described here and the genomic expression of the ev22 and HIV gag retroviral sequences needs further clarification.

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5

RETROVIRAL p15E-RELATED SERUM FACTORS RELATED TO RECURRENCE OF HEAD AND NECK CANCER

M Tas[1], D Laarman[2]*, M de Haan-Meulman[1], AJM Balm[2], GB Snow[2] & HA Drexhage[1]**

1. Department of Immunology, Erasmus University Rotterdam and
2. Department of Otorhinolaryngology, Head and Neck Surgery,
Free University Hospital, Amsterdam
** present address: St Joseph Hospital, Heemskerk*
*** present address: Department of ENT and Head and Neck Surgery,*
The Netherlands Cancer Institute (Antoni van Leeuwenhoek Huis), Amsterdam

SUMMARY

Surgical removal of head and neck squamous cell carcinoma (HNSCC) restores the defective monocyte polarization found in HNSCC patients. Since HNSCC contain p15E-like low molecular weight factors <25kD (LMWF) capable of suppressing N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced monocyte polarization, it is likely that HNSCC removal eradicates the production site of p15E-like factors.

This report describes a prospective follow-up study on the levels of bioactive p15E-like serum factors during a period of two years in nine HNSCC patients who had no recurrence and 11 HNSCC patients who showed residual or recurrent disease after therapy with curative intent. In the group of patients without recurrent disease p15E-like bioactivity gradually decreased and eventually became negative. In the patients with recurrent/residual disease p15E-like bioactivity remained high or even became positive before or at the time of diagnosing tumor recurrence.

This study strongly supports the concept that HNSCC tumors are the production site of p15E-like immunosuppressive factors and indicates that the positivity for serum p15E-like factors may be used for future studies on early serum markers for recurrent/residual disease developing in the first year after treatment.

INTRODUCTION

The transmembrane envelope protein (TM-protein) p15E of animal retroviruses is an important mediator of immune dysfunction associated with animal retroviral infection (1-7). In particular lymphocyte blastogenesis, lymphokine secretion and monocyte chemotaxis are suppressed. Interestingly, various malignant cells produce factors structurally and functionally related to p15E (8). Infection with retroviruses could not be detected in these p15E-related factor-positive tumor cells (8), indicating that the production of these p15E-like factors is due to an abnormal expression of endogenous retroviral-like sequences.

At earlier occasions we reported the presence of p15E-like factors in human head and neck squamous cell carcinomas (HNSCC)(9). Low molecular weight fractions <25kD (LMWF) prepared from HNSCC exerted inhibitory effects on monocyte chemotaxis both *in vitro* and *in vivo* (10,11), and on the swelling of delayed-type hypersensitivity (DTH) skin reactions in mice (12). LMWF prepared from the serum of such patients also inhibited the chemotactic responsiveness of healthy donor monocytes and this inhibition by LMFW was adsorbable by monoclonal antibodies (mAb) to retroviral p15E (13). This could explain the defective chemotactic capability of monocytes from patients with HNSCC by circulating tumor-derived p15E-like LMWF (13). Moreover, surgical removal of tumors associated with p15E-like suppression restores the defective monocyte function. It is likely that this removal eradicates the production site of p15E-like factors (14,15).

This report describes a prospective follow-up study on the bioactivity of p15E-like serum factors during a period of two years in a group of 20 HNSCC patients from

whom nine had no recurrence after treatment, and 11 showed residual or recurrent disease after therapy, i.e. regrowth of their tumor within two years.

MATERIALS AND METHODS

Patients

All patients were treated by surgery and/or by radiotherapy at the department of ENT/H&N surgery of the Free University Hospital, Amsterdam. Series of sera were collected by venipuncture from 20 patients with HNSCC from the day of diagnosis through two years after starting therapy. Nine patients had no residual/recurrent disease (patients 1-9; table 1) and 11 patients showed residual/recurrent disease (patients 10-20; table 1). These sera had been stored at -70°C and were coded before testing.

Healthy controls

Sera obtained from 79 healthy controls (aged 22-67 years) were stored at -70°C until tested.

LMWF

Sera from the healthy controls and patients were thawed and diluted 1:1 in saline. These dilutions were subjected to ultrafiltration through Amicon CF25 Centriflo cones (Amicon Corp., Danvers, USA) for 15 min at 800 g (molecular weight 'cut off point' 25 kD). The residues were resuspended and stored at -20°C until further use.

Adsorption procedures

To validate the p15E-like bioactivity of the LMWF, adsorption experiments were carried out by reacting the serum fractions, before testing the effects on monocyte polarization, with a combination of two p15E specific mAb (see below) in a final dilution of 1:200 (final IgG concentration 50 μg/ml) at 4°C for 16 h, followed by Amicon ultrafiltration to remove formed complexes. This adsorption procedure was carried out twice (13). The mAb used were a combination of 4F5 and 19F8 (anti-p15E isotypes IgG2a and IgG2b; kindly provided by Dr. G.J. Cianciolo, Genentech Inc., Pharmacological Sciences, South San Francisco, CA, USA). As control antibodies we used irrelevant anti-human IgG2a (ICN 64-336, ICN Biomedical Inc., Costa Mesa, CA, USA) and IgG2b (ICN 64-337).

Monocyte isolation

Monocytes from healthy donors were isolated via counterflow elutriation centrifugation. These monocytes were used to test the effects of the LMWF on the polarization of healthy donor monocytes. In brief, mononuclear cells were separated from 450 ml whole blood of the healthy controls via Percoll centrifugation (20 min, 1000 x g, room temperature). Thereafter, the mononuclear cells were injected into an elutriation centrifugation system (Beckman J21 centrifuge with a JE-6 elutriation rotor). The elutriation medium was PBS with 13 Mm trisodium citrate and 5 mg of human albumin per ml. To separate the different cell populations, the flow rate was kept constant at 20 ml/min, while the rotor speed decreased from 4000 to 0 rpm. The fraction collected at 2500 rpm contained 93-97% monocytes as judged by positivity for non-specific esterase (NSE) activity. This fraction was used in further experiments after storage in liquid nitrogen.

Determination of p15E-like bioactivity

The capability of the serum fractions to inhibit N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced polarization of healthy donor monocytes was determined as follows: Monocytes were thawed and resuspended in RPMI 1640 supplemented with 10% fetal calf serum (GIBCO, Breda, The Netherlands), 2mM glutamine and antibiotics (medium) and washed twice. 0.2 x 10^6 monocytes were added to 12-75 mm polypropylene tubes (Falcon Labware Division of Becton Dickinson Co., Oxford, CA,

Table 1. Clinical data and bioactivity of p15E-like factors.

	m/f	age	site	stage	histol. grade**	bioactivity p15E-like factors before treatment	surgical treatment	radio-therapy	chemo-therapy	bioactivity p15E-like factor 0-5 months	6-11 months	12-17 months	18-23 months	time interval after treatment and diagnosed recurrent disease
Group A														
1.	♂	70	larynx	T3N0	m	11%	+	—	—	16%	18%	28%	14%	—
2.	♀	62	oral cavity	T4N0	m	5%	+	—	—	2%	5%	16%	—	—
3.	♂	56	oropharynx	T3N1	m	23%	+	40 days	—	17%	9%	4%	7%	—
4.	♀	57	oropharynx	T3N1	m	0%	+	45 days	—	22%	0%	—	6%	—
5.	♂	74	larynx	T4N0	m	1%	—	45 days	+	1%	1%	1%	4%	—
6.	♂	57	oral cavity	T3N0	p	24%	+	—	—	10%	0%	—	—	—
7.	♂	58	oral cavity	T3N2	w	15%	+	—	—	0%	6%	—	0%	—
8.	♂	51	oral cavity	T3N2	m	34%	+	135 days	—	0%	8%	—	0%	—
9.	♂	68	larynx	T4N3	w	30%	+	45 days	—	6%	4%	0%	—	—
Group B														
10.	♂	60	oral cavity	T4N1	m	8%	+	35 days	—	20%	25%*			6 mths
11.	♂	51	oropharynx	T3N2	m	3%	+	60 days	—	8%	6%	29%	17%*	20 mths
12.	♂	57	larynx	T2N0	ND	15%	—	50 days	—	8%*				6 mths
13.	♂	50	oral cavity***	T3N1	m	28%	—	60 days	+	—	17%*			6 mths
14.	♂	58	larynx	T2N0	ND	23%	—	50 days	—	10%	18%*			7 mths
15.	♂	52	larynx	T2N0	p	21%	—	60 days	—	24%	28%*			6 mths
16.	♀	48	oropharynx	T4N0	m	36%	—	60 days	+	18%	16%	15%	34%*	22 mths
17.	♂	81	larynx	T3N0	m	ND	—	45 days	—	23%	59%*			6 mths
18.	♂	41	oropharynx	T4N2	w	22%	—	30 days	+	52%*				3 mths
19.	♂	58	larynx	T3N0	m	37%	+	—	—	58%	32%*			11 mths
20.	♀	86	larynx	T2N0	w	26%	+	45 days	—	18%*				3 mths

* recurrence, ** histological grade: p=poorly; m=moderate; w=well; ND=not done. *** Besides a T3N1 tongue carcinoma there was a small T1N0 carcinoma at another site in the oral cavity.

USA) containing 0.05 ml of either medium or fMLP (final dilution of 10 nM), or with fMLP in combination with serum fraction (final dilution 1:60) before or after adsorption with anti-p15E. All experiments were carried out in triplicate. The tubes were incubated at 37°C in a waterbath for 15 min. The incubation was stopped by addition of 0.25 ml ice-cold 10% formaldehyde in 0.05% PBS (pH 7.2). The cell suspensions were kept at 4°C until counting in a hemocytometer using an ordinary light microscope (magnification 250x). The test was read 'blindly' by two persons; 200 cells were counted from each tube. A cell was 'polarized' if any of the following occurred:

1. elongated or triangular shape
2. broadened lamellipodia
3. membrane ruffling

The percentage of inhibition was calculated as follows:

$$i = (1 - (Ff\text{-}fo/Mf\text{-}fo)) \times 100\%$$

Ff = % monocytes polarized after incubation with fMLP and LMWF
Mf = % monocytes polarized after incubation with fMLP alone
fo = % spontaneously polarized monocytes

Addition of serum fractions to non-fMLP stimulated donor monocytes did not affect the spontaneous polarization.

Statistical analysis

At all points after therapy we estimated the relationship between the presence/absence of p15E-like bioactivity in the serum and the presence/absence of residual/recurrent disease by means of logistic regression analysis with a random effect. The random effect was included in the model because the data set consisted of several equidistant time intervals per patient.

Statistical differences between p15E-like bioactivity levels were determined by the paired Student's t-test.

RESULTS

Bioactivity of p15E-like factors

Table 1 shows, besides patients' and tumor characteristics, the results of the bioactivity in serum of p15E-like factors of all these patients during a maximum two years follow-up period. Since the sera were not collected on fixed points in time, we have chosen to give the p15E-like serum activities in half-year time intervals. The actual p15E-like bioactivity is expressed as the percentage inhibition of polarization of healthy donor monocytes due to factors adsorbable with mAb to p15E (see Materials and methods).

Group A consisted of nine patients (no. 1-9) without residual/recurrent disease within two years after treatment. Group B consisted of patients with residual/recurrent disease; nine patients started with primary tumors (no. 10-18), and two patients with operable recurrent malignant disease (no. 19 & 20). Serum was collected from these patients by venipuncture from the day of treatment till the time recurrent/residual disease was diagnosed. Sera were considered to be positive for p15E-like factors if the bioactivity exceeded 17% inhibition of monocyte polarization; this value is based on the bioactivity of 79 healthy individuals (mean (5%) and two times s.d. (6)).

The patients of group A (except for no. 1), who remained tumor free showed a gradual decrease of mean p15E-like factor activity ($p<0.05$, paired Student's t-test) becoming all negative at the last time point tested.

With regard to group B three patients were negative for p15E-like bioactivity before treatment, whereas seven patients were positive. Of one patient (no. 17) the initial p15E-like bioactivity could not be performed. These seven patients were also positive for p15E-like bioactivity before or at the time recurrent/residual disease was diagnosed. In general the p15E-like bioactivity in the serum of patients of group B remained high in follow-up in contrast to the patients of group A. One of the three patients of group B (no. 12) was negative before treatment and remained negative after treatment, in spite of recurrent disease (figure 1). Interestingly, the two patients of group B (no. 10 & 11) who were negative for p15E-like bioactivity before treatment became positive at the time recurrent disease was diagnosed (table 1).

p15E-like bioactivity could neither be related to tumor site, nor to TNM stage or histological grade.

Diagnosable recurrent/residual disease related to the level of p15E-like bioactivity

In figure 2 the probability of diagnosed recurrent/residual disease, given the level of p15E-like bioactivity, is expressed. The data obtained after treatment of all 20 patients were used. We estimated the relationship between the level of p15E-like bioactivity and residual/recurrent disease by means of logistic regression analysis with a random effect. A random effect was included in the model because the data set consisted of several equidistant time intervals per patient.
The model is specified as follows:

$$\text{Prob(recurrence/p15E)} = \frac{1}{1 + \exp(-(\beta_0 + \beta_1 + \text{p15E} + u))}$$

where u is the random effect term and β_0 and β_1 are the coefficients to be estimated. The estimates are given in table 2.

Based on this preliminary set of experiments, 30% inhibition of healthy donor monocyte polarization due to p15E-like factors, already gives a probability of diagnosable recurrent/residual disease of 50% at that moment in this group of patients (figure 2).

Table 2. The estimates of the coefficients for figure 2.

coefficient*	estimates	SE	p
β_0	-3.313	0.914	<0.001
β_1	0.1136	0.0408	0.005

* For the model the coefficients belong to, see results.

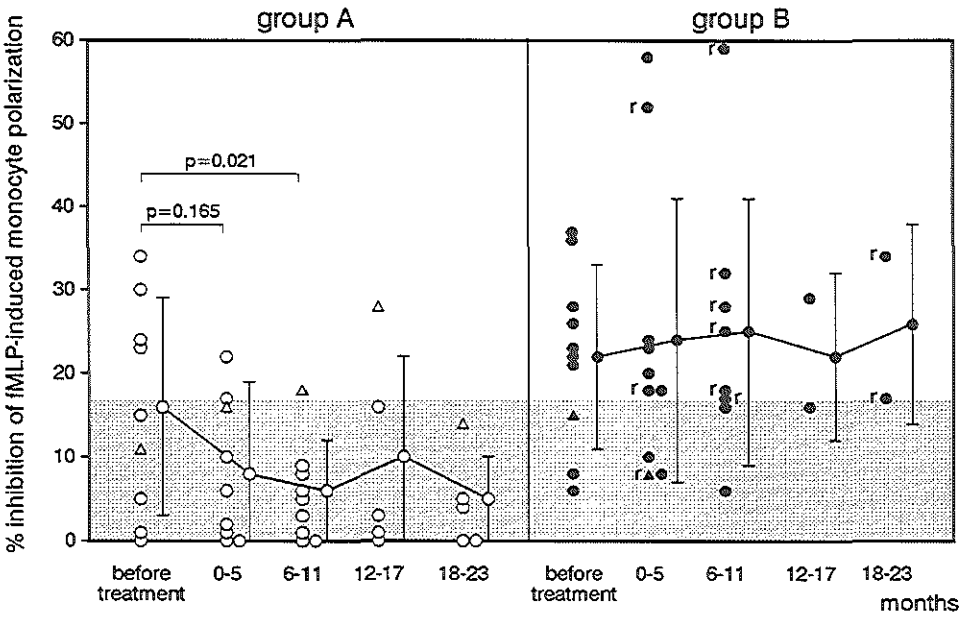

Figure 1. The bioactivity in serum of p15E-like factors of HNSCC patients during a two years follow-up period. The actual p15E-like bioactivity in half-year time intervals is expressed as the percentage inhibition of polarization of healthy donor monocytes due to factors adsorbable with mAb to p15E (see Materials and methods). Group A: patients without recurrent/residual disease; group B: patients with recurrent/residual disease; r = clinically diagnosable recurrent/residual disease; △ = patient with chronic infections of the airways; ▲ = patient who remained negative for p15E-like bioactivity. P-values are given (paired Student's t-test).

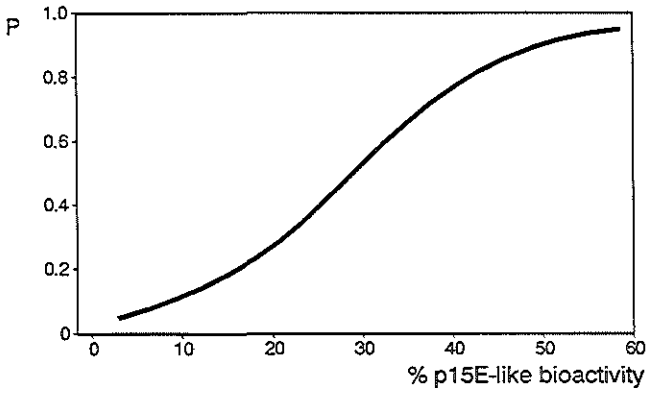

Figure 2. The probability of diagnosable recurrent/residual malignant disease (P), given the level of p15E-like bioactivity (a logistic regression analysis, see text).

DISCUSSION

This study demonstrates that the p15E-like bioactivity of serum decreases to normal values in nine HNSCC patients who remained tumor free after treatment. Furthermore, it shows that the presence of p15E-like factors in serum after treatment is closely related to clinically diagnosable malignant disease in another group of 11 patients.

It can be questioned if p15E-like bioactivity in serum may be used as an early marker of recurrent/ residual HNSCC. Interestingly, eight out of 19 patients were positive for p15E-like bioactivity (i.e. >17% inhibition of monocyte polarization) in the 0-5 months time interval after treatment. In six of the eight patients recurrent/residual disease was clinically diagnosable in 3 to 11 months after treatment. Out of the 11 negative patients only two developed recurrent disease in a 6-11 month period after treatment. This indicates that p15E-like bioactivity harbours the potential of an early marker for recurrent/residual disease. If serum becomes positive for p15E-like factors in the 0-5 months time interval after treatment, chances of getting recurrent or having residual disease in the first year are 75% in our study group; if negative for this factor in this period, chances are only 18%.

With regard to the false negatives and false positives in this series: in one recurrent patient of group B p15E-like bioactivity was never detectable, whereas in group A only one non-recurrent patient became positive during follow-up. In respect to false positive data, it is important to notice that raised p15E-like serum levels are not specific for HNSCC patients, since these suppressive factors can also be found in the serum of patients with defects in cell mediated immunity (CMI) and chronic purulent infections of the upper airways (16), and patients with autoimmune disease, such as Graves' diseases and type1 diabetes mellitus (17) (see also the discussion of this thesis). In this respect it is relevant to mention that the non-recurrent patient of group A with the high p15E-like bioactivity of serum suffered from chronic airway infections.

In conclusion, this study supports the concept that HNSCC is the production site of p15E-like factors, and that circulating p15E-like serum factors may have some predictive value for recurrent disease. A larger prospective study may confirm these data and we are in the process of developing software for standard determination of monocyte polarization. Our latest finding that urine p15E-like factor levels correlate with the serum levels points to a possibility that such a study is also feasable on the urine of patients.

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6

BENEFICIAL EFFECTS OF THE THYMIC HORMONE PREPARATION THYMOSTIMULIN IN PATIENTS WITH DEFECTS IN CELL-MEDIATED IMMUNITY AND CHRONIC PURULENT RHINOSINUSITIS

A DOUBLE-BLIND CROSS-OVER TRIAL ON IMPROVEMENTS IN MONOCYTE POLARIZATION AND CLINICAL EFFECTS*

M Tas[1], JA Leezenberg[2] & HA Drexhage[1]

1. Laboratory for Clinical Immunology, Pathological Institute, and
2. Department of Otorhinolaryngology, Head and Neck Surgery,
Free University Hospital, Amsterdam

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SUMMARY

Twenty patients with chronic purulent rhinosinusitis (CPR), were treated with TP-1 (Serono; 1 mg/kg body weight), in a double-blind cross-over trial. TP-1 was administered by daily i.m. injections for the first 14 days followed by two injections/week for 6 further weeks. The patients were immunologically special in that they had defects in their cell-mediated immune system. Fourteen showed a decreased chemotactic responsiveness of their peripheral blood monocytes as measured in the polarization assay. This defective function can likely be ascribed to the presence in serum of low molecular weight factors (LMWF; <25kD). As reported earlier, this factor shows a structural homology to the envelope protein of murine and feline leukemia virus (p15E). Thirteen patients showed a defective delayed-type hypersensitivity (DTH) skin test reactivity towards candidin and/or streptokinase-streptodornase (SKSD) antigen, 14 had a defective macrophage migration inhibition factor (MIF)-production from their peripheral blood lymphocytes towards candidin, SKSD and/or *Haemophilus influenzae* antigen.

Eighteen patients completed the TP-1 trial and showed clinical improvements: 12 out of 15 were feeling better during TP-1 therapy and the nasal mucosa showed on inspection absent mucopurulent secretion in 13 of the patients. Positive bacterial culture rates for the nose decreased from 14 out of 16 to five out of 15. Placebo treatment had no significant effects.

The clinical improvements were accompanied by a better performance of the cell-mediated immune system; the most significant effects were recorded in the monocyte polarization assay. The suppressive p15E-like LMWF in serum clearly decreased during TP-1 treatment. *In vitro* TP-1 neutralized the immunosuppressive effect of the LMWF. The restoring effects of TP-1 on monocyte polarization and its neutralizing activity of p15E-like LMWF could explain the beneficial effects of thymic hormone treatment reported in adults with clinical signs of immunodeficiency in the presence of a full T cell repertoire.

INTRODUCTION

A disorder frequently encountered in ENT and pediatric practice, is a relapsing of chronic purulent rhinosinusitis (CPR). The pathogenic factors and treatment modalities of this disorder are at present not entirely clear. Surgical interventions to improve the drainage of the sinuses and frequent courses with antibiotics are normally given, but it is our experience that in about 15% of the patients complaints of general malaise, nasal blockage, purulent nasal discharge and headache remain.

As reported earlier in detail, 60% of patients with rhinosinusitis refractory to the current therapies show a defective cell-mediated immunity (CMI) towards the commensal micro-organisms (1).

At first we detected T cell impairments in the patients using delayed-type

hypersensitivity (DTH) skin testing and a macrophage migration inhibition factor (MIF) assay on peripheral lymphocytes; both tests showed faulty reactions towards a panel of microbial antigens (1-4). Other T cell functions, viz., the blastogenic responsiveness towards these antigens and the number of peripheral T cells, were normal (4).

Later we found that an impaired polarization of monocytes accompanied the T cell defects; polarization of monocytes towards chemoattractants is an early event that precedes their chemotactic responsiveness (5). The defective monocyte polarization could be explained by a factor detectable in the patient serum: addition of serum fractions of <25kD to healthy donor monocytes resulted in an inhibition of polarization. The suppressive factor appeared to be p15E related, since monoclonal antibodies (mAb) directed against p15E neutralized the inhibitory effect (6). p15E is the transmembranous part of the envelope protein gp85 of murine and feline leukemia virus and is responsible for the immunosuppressive effects of these retroviruses in their hosts (7).

p15E-related low molecular weight factors capable of inhibiting the function of monocytes have been reported earlier in detail in other patient groups characterized by defects in CMI. Increased levels have been documented in patients suffering from several types of tumors (8-16) and have also been detected in sera of HIV-seronegative homosexuals (17).

The origin of the p15E-related factors in the serum of patients is unknown. The speculative possibilities range from an exogenous infection with an unknown retrovirus producing proteins that share structural homology with p15E, to an endogenous production of the immunosuppressive factor; p15E-related factors were detectable in squamous cell carcinoma's, in epithelial cells overlaying areas of inflammation and in normal thymic epithelial cells (18).

Physiological substances known to have strong effects on the functioning of the CMI are the so-called thymic hormones. In neonates they induce a terminal differentiation in T cells, and are able to stimulate T-lymphocytes to produce lymphokines (19-22). In adults, the administration of thymic hormones has also been described as being effective and low levels of endogenous thymic hormones can be found in several secondary immunodeficiency states, as well as in viral infections and other states of impaired adult host defence (23-25).

This report describes the effects of a double-blind cross-over trial with the thymic hormone preparation thymostimulin (TP-1, Serono) in 20 patients characterized by the above described impairments of CMI and CPR. The *in vitro* effects of TP-1 on the *in vitro* activity of p15E was also tested.

MATERIALS AND METHODS

Patients

Twenty patients, 17 women and three men, aged 23 to 71 years, median 44 years, with a relapsing of purulent rhinosinusitis were included in a double-blind cross-over trial with TP-1. Criteria for inclusion in the study were (1,2):

I. a defective CMI as indicated by

(a) a defective monocyte polarization at previous testing, and/or
(b) a defective skin test to candidin, streptokinase-streptodornase (SKSD) and/or *H. influenzae* antigen at previous testing, and/or
(c) a defective MIF-production towards candidin, SKSD and/or *H. influenzae* at previous testing.

II. relapsing of chronic purulent rhinosinusitis as indicated by
(a) duration of the disease of at least 18 months,
(b) a positive culture for *H. influenzae*, *S. pneumoniae*, other streptococci, or staphylococci at one or more occasions,
(c) no response to or only temporary relief after treatment with several courses of antibiotics,
(d) failure of surgery to give a permanent cure by improving the drainage of the ethmoidal and maxillary sinus,
(e) no gross disturbances in mucociliary transport (exclusion of Kartagener and related syndromes; 26).

All patients had normal levels of total serum IgG, IgM and IgA, normal total numbers of peripheral blood leukocytes and a normal differential lymphocyte count.

A treatment with antibiotics and/or other drugs known to influence the immune system was not given during the TP-1 trial.

Bovine thymic extract (thymostimulin, TP-1)

Thymostimulin is a bovine thymic extract prepared by Serono, according to Bergesi & Falchetti (27) and Falchetti et al. (20), using the following procedures. Calf thymus glands were minced and extracted with ammonium acetate. The extract was heated to 70°C, filtered, and precipitated with ammonium sulfate. The precipitate was dissolved in water and subjected to ultrafiltration on an Amicon PM-10 membrane. The filtrate was desalted on Sephadex G-25 and gel-filtered on Sephadex G-50. The fractions which show two characteristic bands on polyacrylamide gel electrophoresis with Rf 0.22 and 0.24 were combined and termed thymostimulin (TP-1). The extract was lyophilized and its activity expressed as units of T cell rosette formation per milligram of protein. It did not contain endotoxin. In toxicological studies, the extract did not cause any toxic or other side-effects in doses up to 100 mg/kg when administered to mice for 21 days, to rats for 31 days or to cats or dogs for 180 days in doses up to 50 mg/kg (20). The extract did not alter neuromuscular transmission either *in vitro* or *in vivo* (27). The preparation has shown beneficial effects in patients with primary cellular immunodeficiency and combined immunodeficiency (28-31). TP-1 is commercially available in Italy, Spain and Germany.

Double-blind cross-over treatment with thymic factor TP-1 and placebo

Patients gave informed consent and the ethical committee of the hospital had approved the double-blind trial. The treatment consisted of an intramuscular injection of either TP-1 (1 mg/kg body weight) or its placebo (solvent (pyrogen-free sodium chloride solution) + carrier (mannitol); specifically prepared by Serono). Patients were randomly allocated to two groups; one group started with TP-1 injections, the other with placebo injections. Treatment was given for 8 weeks. For the first 2 weeks injections were given daily, followed by twice a week for 6 weeks. On week 9 no treatment was given to test patient's CMI. Thereafter the schedule was crossed over (TP-1 to placebo, and placebo to TP-1) and treatment was given for 8 more weeks.

The week prior to the trial the following tests were carried out in each patient (for techniques see below): (1) an inspection of the nose, (2) a bacterial culture from the nose, (3) a erythrocyte sedimentation rate (ESR), (4) DTH skin tests with bacterial antigens, (5) lymphocyte subset determinations in the peripheral blood, (6) a MIF-assay, (7) a polarization assay with peripheral blood monocytes, (8) a determination of the p15E-like factor in serum.

Tests 1, 2, 3, 7 and 8 were repeated at week 4, 8, 13 and 17 (end of trial). Tests 4, 5 and 6 were only repeated at week 8 (before cross-over) and at week 17 (end of trial).

In all patients parameters relevant to liver function (bilirubin, alanine acid transferrin (ALAT),

aspartic acid transferrin (ASAT)) and kidney function (creatinine, proteinuria) were checked during treatment.

DTH skin tests

The following skin test antigens were used (see also ref. 2): (a) 250 μg/ml of a somatic *H. influenzae* antigen, prepared as described elsewhere (1,3); (b) two commercially available preparations; viz., 1% candidal antigen (HAL allergens, Haarlem, The Netherlands) (= candidin); and 100 U/ml SK and 50 U/ml SD (Varidase, Lederle, Wayne, M, USA).

Delayed responsiveness was tested by intradermal injection of 0.1 ml suspension of each antigen preparation in the forearm. The skin reactions were read at 30 min, 6, 24 and 48 h and the diameter of the induration, expressed as the average of two measurements at right angles, was recorded.

Enumeration of total peripheral blood lymphocytes and lymphocyte subsets

The percentage of lymphocytes and lymphocyte subsets was determined by reacting peripheral blood lymphocytes isolated by Ficoll-Isopaque density gradient centrifugation (Pharmacia, Diagnostics AC, Uppsala, Sweden) with $CD3^+$ antibodies against T cells (Leu 4; Becton Dickinson, Mountain View, CA, USA), $CD2^+$ antibodies against active T cells (OKT 11; Orthoclone Ortho, Raritan, NJ, USA), $CD4^+$ helper/inducer T cells (3a), $CD8^+$ suppressor/cytotoxic T cells (Leu 2a), and $CD24^+$ B cells (BA-1; Hybritech Inc., San Diego, CA, USA) as indicated by the manufacturer. Two-hundred cells were counted in a fluorescent microscope; the tests were done in duplicate. Absolute numbers of T cells and T cell subsets were calculated by multiplication from the total peripheral lymphocyte counts.

Macrophage migration inhibition factor test

MIF production was estimated with an indirect microdroplet agarose assay as described in detail by van de Plassche-Boers et al. (4). In brief, peripheral blood mononuclear cells (2.5×10^6) were cultured with the antigens of *H. influenzae*, candidin and SKSD. Supernatants were also prepared using the mitogen Concanavalin A (ConA, Sigma, St Louis, MO, USA). Supernatants were collected after 3 days of culture (37°C, 5% CO_2 in air) and stored at -20°C until testing for MIF activity.

The agarose microdroplet assay was performed according to Thurman et al. (32) using the human monocytoid U937 as indicator cells (33). From the cells (2×10^7 cells/ml) in 0.2 % agarose (Marine Colloids, Rockland, USA) 1 μl droplets were centrally placed in the wells of flat-bottomed microtiter plates (Nunc, Denmark) using a Hamilton repeating dispenser with a 0.05 ml gas-tight syringe (Hamilton, Reno, AR, USA). The droplets were left to solidify at 4°C for 10-20 min, and carefully overlaid with 0.1 ml of thawed supernatant diluted 1:1 with fresh medium. Each supernatant was tested five times. After incubation of the covered plates for 21 h at 37°C, and 5% CO_2 in air, migration areas (cell migration area minus area of the agarose droplet) were computed using a projection microscope and a graphic tablet, connected to a computer. MIF production was expressed as per cent migration inhibition:

$$MI = 100 - \frac{\text{mean migration area in antigen-stimulated cultures}}{\text{mean migration area in medium}} \times 100\%$$

The isolation of peripheral blood monocytes and the polarization assay

Peripheral blood mononuclear cells (20×10^6) isolated by Ficoll-Isopaque density gradient centrifugation were washed twice in phosphate buffered saline (PBS), pH 7.4 containing 0.5% bovine serum albumin (BSA), and counted in suspension employing positive staining with Non Specific Esterase (NSE; 34). The percentage of NSE-positive cells varied, at 5-25%. An enrichment for the monocytes in the Ficoll-Paque isolated fraction was obtained by Percoll gradient centrifugation (35). After washing, the pellet containing the monocytes was resuspended in the above-mentioned medium and carefully underlayed with an equal volume of Percoll 1.063 (Pharmacia, Diagnostics AC, Uppsala, Sweden). After centrifugation (40 min, 450 g) the cells were collected from the interface, washed twice in medium (10

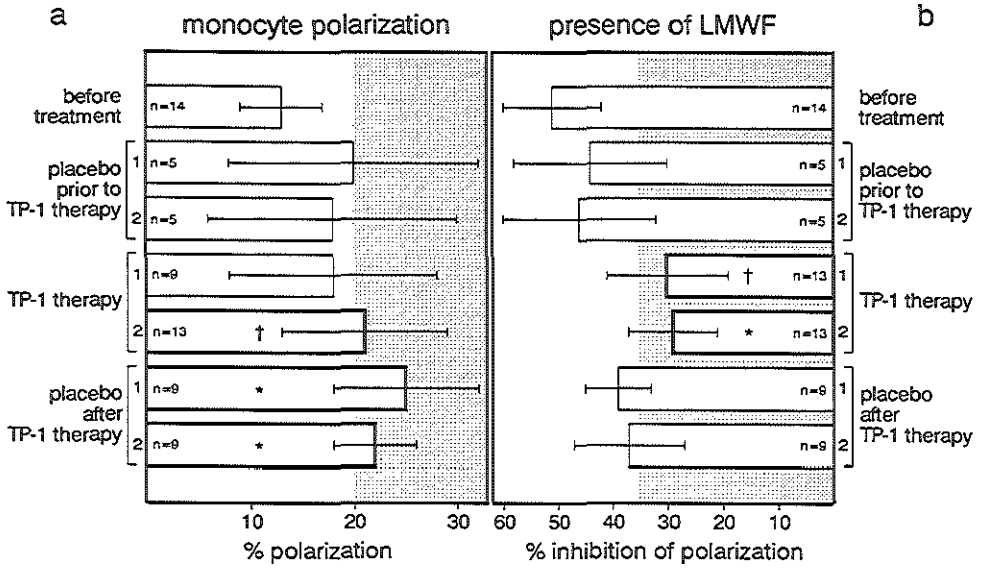

Figure 1. Monocyte polarization (a) and inhibition of polarization (b). **a.** fMLP-induced polarization of patients' monocytes. Fourteen out of 18 patients had a polarization lower than normal before any treatment. **b.** The activity of serum fractions <25kD (LMWF) from these patients expressed as the inhibition of fMLP-induced polarization of healthy donor monocytes. Monocytes were obtained via Ficoll/Percoll gradient centrifugation. The hatched areas represent the values found in healthy controls. Statistically significant differences are given (* $p<0.01$, † $p<0.05$; Wilcoxon).

min, 500 g) and counted: the suspension now contained 70-95% NSE-positive cells.

The Cianciolo and Snyderman (5) assay for monocyte polarization was performed with slight modifications (15). 0.2 ml aliquots of the Percoll- or elutriator-purified cell suspension containing 0.2 x 10^6 monocytes were added to 12-75 mm polypropylene tubes (Falcon Labware Division of Becton Dickinson Co., Oxford, CA, USA) containing 0.05 ml of either medium or N-formyl-methionyl-leucyl-phenylalanine (fMLP) in medium, to reach a final concentration of 10 nM. All experiments were carried out in duplicate. The tubes were incubated at 37°C in a waterbath for 15 min. The incubation was stopped by addition of 0.25 ml ice-cold 10% formaldehyde in 0.05% PBS (pH 7.2). The cell suspensions were kept at 4°C until counting in a hemocytometer using an ordinary light microscope (magnification 250x). The test was read 'blindly' by two persons (figure 1); 200 cells were counted from each tube. A cell was 'polarized' if any of the following occurred:

1. elongated or triangular shape
2. broadened lamellipodia
3. membrane ruffling.

The percentage of polarized monocytes was calculated as follows:

$$\frac{\%\text{ total cells polarized}}{\%\text{ NSE-positive cells}} \times 100\%$$

Lymphocytes do not exhibit any polarization activity in this assay (5).

The chemotactic responsiveness of a monocyte population is expressed as the percentage of polarized monocytes in the presence of fMLP minus the percentage of polarized monocytes in the absence of fMLP. The assay has proven to be a rapid method to test monocyte chemotaxis and outcomes of the assay correlate well with outcomes of the conventional Boyden chamber assay to measure chemotaxis to casein (15). Of 77 healthy control individuals, a chemotactic responsiveness was found of 33 (mean, s.d. 11; ranges from 18 to 70), there were no differences between female and male individuals (mean 33, s.d. 9 (n=36); or 32, s.d. 13; n=41) or between individuals less than 50 years and over 50 years of age (mean 34, s.d. 11 (n=66); or 31, s.d. 8; n=11). When one monocyte population was tested several times (inter-assay variation) outcomes were 24 (mean, s.d. 3; n=13). The intra-assay variation also never exceeded 15% (n=77). On the basis of these outcomes fMLP induced polarization values of less than 20% are considered to be abnormal.

The determination in patient serum of low molecular weight factors (LMWF) inhibiting monocyte polarization

Sera were collected from the patients by venipuncture and diluted 1:1 in saline. These dilutions were subjected to ultrafiltration through Amicon CF25 Centriflo cones (Amicon Corp., Danvers, USA) for 15 min at 700 g (molecular weight 'cut off point' 25kD). The residues were resuspended and stored at -70°C until further use.

The capability of the serum fractions to inhibit fMLP-induced polarization of healthy donor monocytes was determined by incubating the monocytes (1×10^6/ml) during 15 min at 37°C either with fMLP alone or with fMLP in combination with a serum fraction (final dilution 1:60).

Addition of serum fractions alone to donor monocytes did not affect the polarization. The percentage of inhibition of fMLP-induced minus spontaneous polarization caused by addition of the serum fractions was calculated.

The *in vitro* effects of anti-p15E mAb and TP-1 on the activity of serum LMWF

In this series of experiments, elutriator-purified monocytes (36) of one healthy donor were used as an indicator system. In brief, mononuclear cells were separated from 450 ml whole blood via percoll centrifugation (20 min, 1000 g, room temperature). Thereafter the mononuclear cells were injected into an elutriation centrifugation system (Beckman J21 centrifuge with a JE-6 elutriation rotor). The elutriation medium was PBS with 13 mM trisodium citrate and 5 mg of human albumin per ml. To separate the different cell populations, the flow rate was kept constant at 20 ml/min while the rotor speed was diminished from 4000 to 0 rpm. The fraction at 2500 rpm was collected. After Percoll gradient centrifugation this fraction contained 93-97% monocytes as judged by positivity for non-specific esterase activity. Monocytes were stored in liquid nitrogen until use.

With this indicator system the p15E-like character of the LMWF was validated. Adsorption experiments were carried out by incubating the serum fractions with a combination of two p15E specific mAb (see below) in a final dilution of 1:200 at 4°C for 16 h, followed by Amicon ultrafiltration to remove formed complexes; this adsorption procedure was carried out twice (16). The mAb used were a combination of 4F5 and 19F8 (anti-p15E isotypes IgG2a and IgG2b; kindly provided by Dr. G.J. Cianciolo, Genentech Inc., Pharmacological Sciences, CA, USA). As a control antibody we used an anti-human IgG (TAGO Inc., Burlingame, CA, USA).

Patient serum fractions of <25kD diluted in culture fluid or incubated with TP-1 (final dilution 67 μg/ml) and serum fractions after adsorption with the mAb were tested in the monocyte polarization assay.

RESULTS

Of the 20 patients enrolled in the double-blind cross-over trial 18 completed the

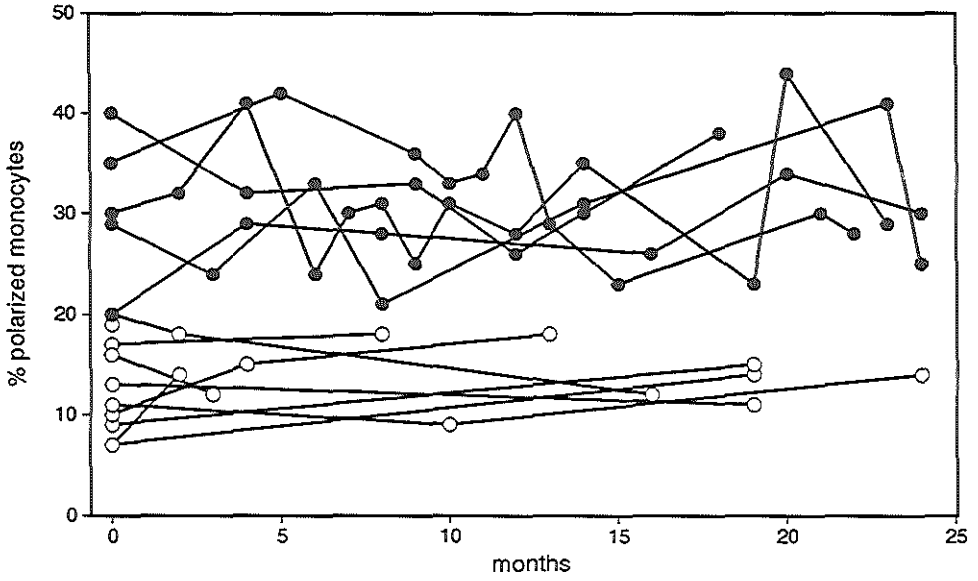

Figure 2. The fluctuations in time of fMLP-induced monocyte polarization. ● healthy controls; ○ patients without TP-1.

17 weeks course of treatment. One patient (a female, 23 years old) was removed from the trial because she reacted with fever, nausea and malaise to the injections, an allergy to calf thymic peptides was established by a positive skin test to the preparation. The other patient (a female, 53 years old) was wrongly enrolled in the trial; she had no CMI defects detectable before treatment. The 18 patients who completed the trial did not have clinically adverse side effects of the treatment, biochemical blood changes such as abnormalities in the level of bilirubin, ALAT, ASAT or creatinine were also not detectable.

When we decoded the patients at the end of the double-blind cross-over trial it appeared that seven patients had been allocated to the group receiving placebo prior to TP-1 therapy, the remaining 11 patients had received TP-1 first. This left us with four situations to be evaluated:

1. performance before any treatment (all 18 patients);
2. performance during and after placebo treatment prior to TP-1 therapy (seven patients);
3. performance during and after TP-1 therapy (all 18 patients);
4. performance during and after placebo treatment after TP-1 therapy (11 patients).

Monocyte polarization

With regard to the polarization of the patients monocytes, 14 of the 18 patients included in the trial had a polarization lower as normal before treatment. The mean

value found in these 14 patients was 13% (s.d. 4%; figure 1a). Normal values in healthy controls are over 20% (see Materials and Methods). Figure 2 shows the fluctuations in time of the monocyte chemotaxis measured by polarization: when healthy donors are followed up to 2 years, fluctuations range from 20 to 45%. In a series of experiments prior to this trial, 10 patients were left untreated; only one showed values of 19 to 22% in 3 months time, the others did not reach values exceeding the 20% level. In contrast there was a significant rise in the percentage of polarized monocytes in this patient group after 2 months of TP-1 therapy and values of 21% (mean) ± 8% (s.d.) were reached (figure 1a); besides 7 out of 13 patients were now in the range of normal healthy individuals. This restoring effect continued during the placebo period after TP-1 therapy and values of 25% (mean, s.d. ± 7%, 1 month) and 22% (mean, s.d. ± 4%, 2 months) were reached, while 6 out of 9 of the patients were in the normal range. Placebo treatment prior to TP-1 therapy had no significant effects on the defective monocyte polarization.

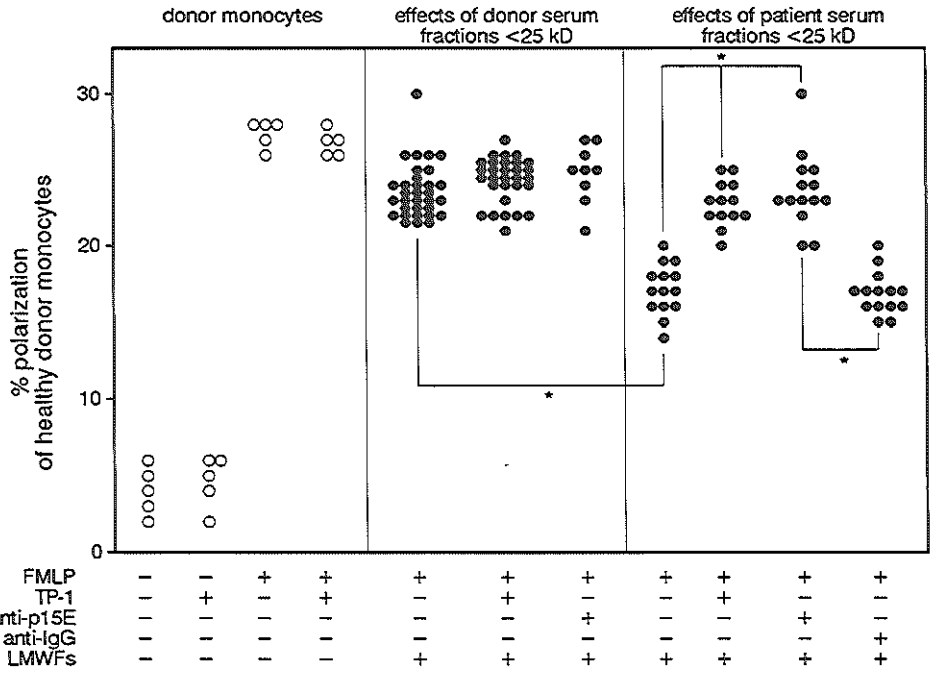

Figure 3. Per cent polarization of elutriator-purified healthy donor monocytes with or without fMLP and the activity of serum fractions <25kD (LMWF) from healthy donors and patients diluted in medium or incubated with TP-1 (final dilution 67 μg/ml) and serum fractions after adsorption with the monoclonal antibodies anti-p15E and anti-human IgG. Statistically significant differences are given (Wilcoxon).

Serum p15E-like factors

Before any treatment, the 14 patients with a defective monocyte polarization had immunosuppressive LMWF detectable in their serum (see figures 1b and 3): serum fractions of <25kD induced a 51% (mean, s.d. ± 9%) inhibition of fMLP-induced polarization of healthy donor monocytes obtained via Percoll gradient centrifugation (figure 1b) and a 45% (mean, s.d. ± 7%) inhibition of fMLP-induced polarization of healthy donor monocytes obtained via centrifugation with an elutriator (figure 3).

Figure 3 shows that the inhibitory effect of patient LMWF on the polarization of healthy donor monocytes could be neutralized by treating the LMWF with a combination of two mAb to p15E. A control antibody had no such effect. This shows the p15E-like character of the LMWF of these patients, as has been established before (6).

After TP-1 therapy patient serum LMWF were no longer detectable and values of inhibition of polarization decreased to 30% (s.d. ± 11%, 1 month therapy) and 29% (s.d. ± 8%, 2 months therapy) respectively (figure 1b). LMWF isolated from the serum of healthy individuals also have such low inhibitory effects on healthy donor monocytes (<35% inhibition of polarization of monocytes obtained via Percoll isolation (6) and <30% inhibition of elutriator purified monocytes; (mean 14, s.d. 6; n=34) (figure 3).

Placebo treatment prior to TP-1 therapy had no statistically significant effects on the disappearance of the LMWF from serum, and the LMWF soon reappeared in the placebo period after TP-1 therapy (figure 1b).

In vitro TP-1 also appeared to be able to neutralize the suppressive activity of patient serum LMWF when added to the system of elutriator purified monocytes (figure 3). TP-1 had no direct effect on spontaneous or fMLP-induced polarization of the healthy donor monocytes.

DTH skin testing and MIF production

There were some improvements in DTH skin test reactivity during TP-1 therapy (table 1a). Regarding the candidin skin test only eight out of 18 patients showed a positive DTH test before any treatment, after 2 months of TP-1 therapy 14 out of 15 patients showed a positive according; values stayed high during the placebo period after TP-1 therapy eight out of 10. Placebo treatment prior to TP-1 therapy had no such effects.

Regarding the SKSD skin test, five out of 18 patients had a positive recording before treatment, after TP-1 therapy 10 out of 17 and six out of 10 (post TP-1 placebo period) showed a positive DTH, respectively. However placebo treatment prior to TP-1 therapy had a curious effect; six out of seven patients had a positive recording.

The MIF production towards ConA, *H. influenzae*, candidins and SKSD showed defective responses before treatment (table 1b); only nine out of 18, 10 out of 18 and four out of 18 patients were positive, respectively. Placebo treatment prior to TP-1 therapy had no effect on MIF production. After 2 months of TP-1 therapy there was an increase in responder rates in the case of SKSD seven out of 10 and *H. influenzae* 13 out of 17, the values decreasing again during the placebo period after TP-1 therapy (to

Table 1. (a) Positive DTH skin test reactivities to the antigens *H. influenzae* (25 µl) and candidin (1% solution). The diameter of induration (in mm) was recorded 24 h after antigen injection. Percentages of positive skin tests >20 mm induration) and p-values for statistical differences (χ^2) are given. (b) Positive MIF production to the mitogen ConA and the antigens *H. influenzae*, candidin and SKSD as measured as the macrophage migration inhibition. A percentage inhibition of >25% was considered as positive.

(a)

	DTH skin test	
	candidin	SKSD
Before treatment	8/18	5/18
Placebo prior to TP-1 therapy	4/6	6/7
TP-1 therapy	14/15	10/17
Placebo after TP-1 therapy	8/10	6/10

(b)

	MIF production			
	ConA	H.I.	candidin	SKSD
Before treatment	9/18	9/18	10/18	4/18
Placebo prior to TP-1 therapy	3/6	3/6	2/6	1/6
TP-1 therapy	9/17	13/17	9/17	7/10
Placebo after TP-1 therapy	6/11	6/11	7/11	1/11

* p=0.35; † p+0.003; ‡ p=0.07; § p=0.009; ¶ p=0.06; ** p=0.09.

one out of 11 and to six out of 11, respectively). However, increases in responder rates did not reach statistical significance.

Surface markers

With regard to the number of $CD3^+$ (total T), $CD4^+$ and $CD8^+$ peripheral blood lymphocytes, near normal numbers were found before any treatment, and numbers stayed within these normal limits throughout the period of treatment irrespective of whether this was TP-1 or placebo. However, as noticed earlier (2), there was a lowered CD2 expression on peripheral lymphocytes in 40% of the patients before treatment (<63% of peripheral blood lymphocytes, normal values in our laboratory range from 63-79% of lymphocytes with CD2 expression). At the end of placebo treatment prior to TP-1 therapy, 33% of the patients had a lowered CD2 expression on peripheral lymphocytes, however, at the end of 2 months of TP-1 therapy this value was decreased to 13% of the patients. After TP-1 therapy the phenomenon of the lower expression of the CD2 marker reappeared and now 64% of the patients had diminished percentages of $CD2^+$ lymphocytes.

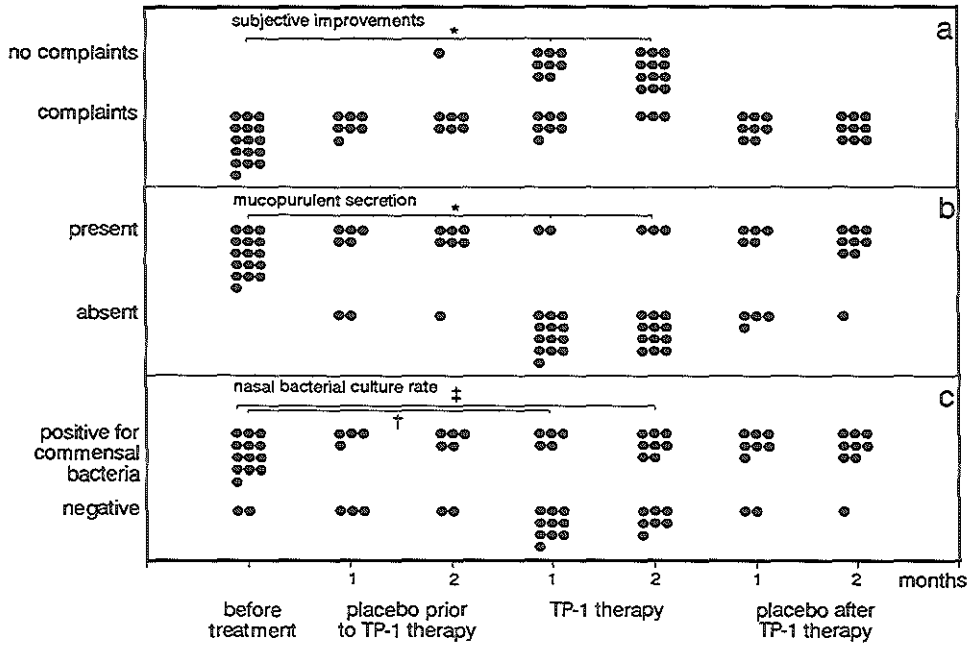

Figure 4. Clinical improvements. (a) Subjective improvements after TP-1 therapy; (b) absence of mucopurulent secretion after TP-1 therapy; (c) the prevalence of positive bacterial culture rate (*H. influenzae*, *Branhamella*, *Klebsiella*, *Moraxella*, *Citrobacter*, Streptococci and/or Staphylococci) decreased after TP-1 therapy. *Statistically significant difference; χ^2; $p<0.001$, † $p<0.002$, ‡ $p<0.05$).

With regard to the peripheral B cells (as measured as $CD24^+$ cells) there was a significant increase in percentages during TP-1 therapy.

Clinical improvements

The clinical condition of the 18 healthy patients showed a considerable improvement during TP-1 therapy: subjectively 12 out of 15 were clearly feeling better during specific therapy (figure 4a) and showed an absent mucopurulent secretion on inspection of the nasal mucosa (figure 4b). Placebo treatment prior to TP-1 therapy had no such effects and rapid relapses were recorded during the placebo period after TP-1 therapy. The prevalence of a positive nasal bacterial culture rate (*H. influenzae*, *Branhamella*, *Citrobacter*, *Klebsiella*, *Moraxella*, *S. pneumoniae*, other streptococci, and/or staphylococci) decreased from 14 out of 16 before treatment to five out of 15 during TP-1 treatment (figure 4c), followed by a rise to again eight out of nine positivity 2 months after TP-1 therapy. TP-1 treatment had no effects on the ESR. Only one of the patients had besides the CMI defects signs of an atopic allergy (raised serum IgE, positive (RAST), asthmatic attacks). TP-1 treatment had no effect on the atopic complaints and IgE levels stayed high.

DISCUSSION

This report shows the beneficial clinical effects of TP-1 treatment in a double-blind cross-over trial on the clinical condition, the purulent nasal discharge and the bacterial invasion of the upper airways in patients with CPR resistant to the current therapies of surgery and antibiotics.

The clinical improvements during TP-1 therapy were accompanied by a raised performance of the CMI of the patients, which probably forms the basis for their improved clinical condition. The most predominant and significant effects were recorded in monocyte chemotaxis as measured in the polarization assay. MIF production by peripheral blood lymphocytes and DTH skin test reactivity to microbial antigens were also enhanced during TP-1 treatment, but in general statistical significance was not reached, except for the candidin DTH skin test.

The number of patients in this double-blind cross-over trial is limited and the period of active treatment (8 weeks) is rather short. However, similar effects were obtained in an open not-controlled trial with TP-1 in 10 patients with CMI defects and a relapsing of CPR (carried out before this double-blind cross-over trial). All treated patients showed clinical improvements: they were feeling better and their nasal mucosa showed less to absent mucopurulent secretion on inspection. The positive bacterial rate decreased from eight out of 10 to three out of 10. The clinical improvements were accompanied by a better performance of their CMI; the clearest effects were again recorded in the monocyte polarization assay (before treatment 10 (mean, s.d. 5; n=8); after 8 weeks of TP-1 treatment 27 (mean, s.d. 16; n=8).

At an earlier occasion we presented evidence that the impairment in monocyte polarization found in our patient group is most probably due to the presence in serum of a LMWF of <25kD showing a structural homology with p15E of murine and feline leukemia virus (6). The data presented in this report further strengthen this view. p15E-like factors were detected in the serum of 14 out of 18 patients before any treatment; these patients also showed an impaired monocyte polarization. There was a functional disappearance or decrease of the activity of the p15E-like factors during TP-1 treatment in the double-blind cross-over trial. In the above mentioned open trial five patients were tested for the presence of the p15E-like factor in serum; in these five patients the factor also disappeared after TP-1 treatment, expressed as inhibition of monocyte polarization: before treatment 52% (mean, s.d. 13), after 8 weeks of TP-1 treatment 19% (mean, s.d. 16). Whether this shows a real molecular disappearance of the factor from the serum of TP-1 treated patients (non-production) or a functional neutralization of the factor by thymic hormones in the serum needs further study. Also, *in vitro* TP-1 neutralized the suppressive activity of patient serum LMWF. TP-1 had no effect on spontaneous or fMLP induced polarization of healthy donor monocytes.

The most well-known function of thymic hormones is their effect on the maturation and differentiation of T cells in the thymic micro-environment. Many studies have focussed on the stimulating effects of TP-1 on T cell maturation and reactivity both in primary as well as in secundary immune deficiency states (37-39). The beneficial

outcomes of TP-1 treatment in primary immunodeficiencies in neonates can be attributed to this effect.

From our data it is, however, tempting to speculate that some of the compounds of the thymic peptide hormone preparation may exert their effect via cells of the mononuclear phagocyte system, thereby indirectly influencing T cell-mediated immunity. Such function could explain the beneficial effects of thymic hormone preparations seen in adults with secundary immunodeficiencies and a full T cell repertoire. Reports on the effects of thymic hormones on the functioning of cells of the mononuclear phagocyte system are, however, extremely scarce. Davis et al. (40) reported an effect of TP-1 treatment on serum lysozyme levels in patients with Hodgkin's disease, which suggested an increase in monocyte function and/or mass. There is also a report on a disturbed monocyte natural cytotoxicity in viral hepatitis, which function became normal after *in vitro* incubation of the monocytes with thymic preparations (41). Zatz et al. (42) described that the augmentation of IL-2 production of peripheral blood leukocytes induced by thymosin fraction 5 was prevented by monocyte depletion. They interpreted their data as demonstrating that the thymic hormone effect in lymphocyte IL-2 production was mediated directly or indirectly via a monocyte population. This view that TP-1 may also act on accessory cells is further supported by preliminary experiments carried out in our laboratory, which show that p15E inhibited clustering of dendritic cells (a monocyte-like cell) is also restored to normal by the *in vitro* addition of TP-1.

Current treatment protocols of CPR form an agony for both patient and physician (frequent consultations, surgical interventions and several courses of antibiotics). Our data support a further critical evaluation of the use of thymic hormone preparations and justify larger controlled trials. Purification procedures are necessary to determine which thymic hormone fraction is responsible for the neutralizing effects on the activity of p15E-like factors in monocyte polarization.

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7

DEPRESSED MONOCYTE POLARIZATION AND CLUSTERING OF DENDRITIC CELLS IN PATIENTS WITH HEAD AND NECK CANCER

IN VITRO RESTORATION OF THIS IMMUNOSUPPRESSION BY THYMIC HORMONES*

M Tas[1], PJ Simons[1], AJM Balm[2] & HA Drexhage[1]

1. Department of Immunology, Erasmus University, Rotterdam
2. Department of ENT and Head and Neck Surgery, The Netherlands Cancer Institute (Antoni van Leeuwenhoek Huis), Amsterdam

* *Submitted for publication*

SUMMARY

The *in vitro* restoring effects of a thymic hormone preparation, TP-1, on defective monocyte and dendritic cell function in patients with head and neck squamous cell carcinoma (HNSCC) have been examined. The N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced polarization of monocytes isolated from the peripheral blood was significantly lower (a mean of 19%) than the polarization of monocytes isolated from healthy controls (a mean of 33%). After the *in vitro* addition of TP-1 this defective polarization was improved to the normal value of 33% polarized monocytes. The capability of dendritic cells (DC) prepared from the blood to form cellular clusters with allogeneic cells was impaired in 26/44 patients. *In vitro* addition of TP-1 again had restoring effects. The original defective DC clustering of 97 clusters/six microscopic fields (mean) was improved to a value of 121 clusters. The defects in monocyte polarization and clustering of dendritic cells could be ascribed to the presence in serum of a tumor-derived low molecular weight factor (LMWF; <25 kD), sharing structural homology with p15E, the capsular protein of murine and feline leukemogenic retroviruses. The incubation of LMWF from the serum of HNSCC patients with healthy donor monocytes resulted in a significantly higher inhibition of FMLP-induced monocyte polarization than incubation with control LMWF (a mean of 42 vs 16% inhibition). This suppressive effect of patient LMWF was abrogated with a mixture of two monoclonal antibodies (mAb) against p15E as well as with TP-1.

The observations here reported on the *in vitro* effects of TP-1 on depressed monocyte and DC function in HNSCC have provided one of the rationales for a recently started TP-1 therapeutic pilot trial in HNSCC patients.

INTRODUCTION

Monocyte derived macrophages and monocyte derived dendritic cells (DC) are considered to play an important role in the immune reaction against tumor cells. Macrophages have tumoricidal activities when activated by lymphokines produced by tumor-specific T cells (1). DC play a role in the immune response by presenting the tumor-associated antigens to T cells. This latter phenomenon was clearly illustrated by Guyere et al. (2) who showed that effective immunity to a syngeneic sarcoma could be induced in rats by the transfer of very low numbers of dendritic lymph cells exposed to the sarcoma antigens.

Since monocytes, macrophages and DC are capable of anti-tumor activity it is of importance that defects in monocyte and macrophage function have been found in patients with head and neck squamous cell carcinoma (HNSCC). Balm et al. (3) found a clear impairment of the migratory capability of blood monocytes, while Cameron et al. (4) reported a defect in tumoricidal capability of macrophages. The defects in monocyte chemotaxis found by Balm et al. (3) could be ascribed to the presence in serum of a tumor-derived low molecular weight factor (LMWF; <25 kD) capable of

suppressing monocyte polarization, an early event prior to chemotaxis (5). This immunosuppressive factor produced by the tumor shared structural homology with p15E, the capsular protein of murine and feline leukemogenic retroviruses.

Studies on defects and functioning of DC in HNSCC are scarce. Nomori et al. (6) showed that patients with a dense infiltration of DC in nasopharyngeal carcinoma survived longer than those without such infiltration. In the present study the function of DC from the peripheral blood of patients with squamous cell carcinomas at different sites was determined. The most characteristic functional cell biological feature of the dendritic cell is its capability to actively form cellular clusters with immunocompetent cells and creating an optimal microenvironment for antigen presentation and T and B cell activation (7). This physical association of DC with immunocompetent neighboring cells only partly depends on antigen-specific interactions (MHC-antigen-TcR). More important are interactions between adhesion molecules on both sets of cells (8,9,10). In the study reported here peripheral blood DC of HNSCC patients were allowed to form cellular clusters with allogeneic lymphocytes. An impairment of cell clustering was found in comparison to healthy controls.

We reinvestigated the defects in the chemotactic capability of monocytes isolated from the blood of patients with HNSCC (3,11) and the presence of p15E-like factors in the patient serum (5). In earlier studies on chronic purulent rhinosinusitis, positive for the p15E-like immunosuppressive serum factor, thymic hormone preparations (such as TP-1, Serono) abrogated the suppressive effects of the p15E-like factor on monocyte polarization (12). Therefore, we additionally investigated the *in vitro* neutralizing effects of TP-1 on the suppression exerted by p15E-like serum factors of HNSCC patients. The *in vitro* restoring effects of TP-1 on the defective polarization of monocytes and the defective clustering of dendritic cells was also studied.

MATERIALS AND METHODS

Patients

Fifty three patients, 38 males and 15 females, aged 37-78 years (median 59), with head and neck cancer (i.e. squamous cell carcinoma of the oral cavity, larynx and oro- and hypo-pharynx) of stages T1N0-T4N3 were studied.

Healthy controls

Forty three healthy hospital staff members, 20 males and 23 females, aged 23-54 years (median 29), volunteered as controls.

Bovine thymic extract (thymostimulin, TP-1)

Thymostimulin is a bovine thymic extract prepared by Serono, according to Bergesi and Falchetti (13) and Falchetti et al. (14), using the following procedures. Calf thymus glands are minced and extracted with ammonium acetate. The extract is heated to 70°C, filtered, and precipitated with ammonium sulfate. The precipitate is dissolved in water and subjected to ultrafiltration on an Amicon PM-10 membrane. The filtrate is desalted on Sephadex G-25 and gel-filtered on Sephadex G-50. The fractions which on polyacrylamide gel electrophoresis show two characteristic bands with Rf 0.22 and 0.24 are

combined and termed thymostimulin (TP-1). The extract is lyophilized and its activity is expressed as units of T-cell-rosette formation per milligram of protein. It does not contain endotoxin. In toxicological studies, the extract does not cause any toxic or other side-effects in doses up to 100 mg/kg when administered to mice for 21 days or to rats for 31 days, or when administered to cats or dogs for 180 days in doses up to 50 mg/kg (14). The extract does not alter neuromuscular transmission either *in vitro* or *in vivo* (13). TP-1 is commercially available in Italy, Spain and Germany.

The isolation of peripheral blood monocytes

Peripheral blood mononuclear cells (from patients and healthy controls) were isolated by Ficoll-Isopaque density gradient centrifugation. The cells were washed twice in phosphate buffered saline (PBS), pH 7.4 containing 0.5% bovine serum albumin (BSA), and counted in suspension employing positive staining with non specific esterase (NSE; Mullink et al., 15). The percentage of NSE-positive cells varied from 5-25%. An enrichment of the monocytes in the Ficoll-Isopaque isolated fraction was obtained by Percoll gradient centrifugation (Pertoft et al., 16): after washing, the Ficoll-isolated pellet containing both monocytes and lymphocytes was resuspended in RPMI 1640 supplemented with 10% fetal calf serum (GIBCO, Breda, The Netherlands), 2mM glutamine and antibiotics, and carefully underlaid with an equal volume of Percoll 1.063 (Pharmacia, Diagnostics AC, Uppsala, Sweden). After centrifugation (40 min, 400 g) the cells were collected from the interface, washed twice in medium (10 min, 450 g) and counted: the suspension now contained 70-95% NSE-positive cells. This suspension was directly used for the monocyte polarization and maturation of monocytes to obtain dendritic cells (see below).

Monocytes from healthy donors were isolated via counterflow elutriation centrifugation. These almost pure monocyte fractions were used to test the effects of LMWF on healthy donor monocytes and dendritic cells. In brief, mononuclear cells were separated from 450 ml whole blood of the healthy controls via Percoll centrifugation (20 min, 1000 g, room temperature). Thereafter, the mononuclear cells were injected into an elutriation centrifugation system (Beckman J21 centrifuge with a JE-6 elutriation rotor). The elutriation medium was PBS with 13 Mm trisodium citrate and 5 mg of human albumin per ml. To separate the different cell populations, the flow rate was kept constant at 20 ml/min, while the rotor speed decreased from 4000 to 0 rpm. The fraction collected at 2500 rpm contained 93-97% monocytes as judged by positivity for NSE activity. This fraction was used in further experiments after storage in liquid nitrogen.

Metrizamide treatment of monocytes to obtain dendritic cells

Dendritic cells were prepared from peripheral blood monocytes according to the method described by Kabel et al. (17). Metrizamide (Serva, Heidelberg, FRG) was dissolved in RPMI supplemented with 10 % fetal calf serum. Cells from the isolated monocytic fractions were exposed to metrizamide in suspension culture (14.5%) for 30 minutes (5% CO_2 and 37°C, 100% humidity). Thereafter, the cells were washed (culture fluid was added slowly to prevent osmotic lysis of the cells), and further cultured under non-adhering conditions for 16 hours in polypropylene tubes (5% CO_2 and 37°C, 100% humidity). This procedure yields 40-80% cells with a dendritic morphology, showing class II MHC positivity, decreased expression of the monocytic CD14 determinant, decreased phagocytic capability, but enhanced stimulator capability in the mixed leukocyte reaction (MLR). The full technical details of this method are given in Kabel et al. (17).

The monocyte polarization assay

The Cianciolo and Snyderman (18) assay for monocyte polarization was performed with slight modifications (11). The assay has proven to be a rapid method to test monocyte chemotaxis and outcomes of the assay correlate well with outcomes of the conventional Boyden chamber assay to measure chemotaxis to casein (11). 0.2 ml aliquots of the Percoll or elutriator purified cell suspension containing 0.2×10^6 monocytes were added to 12-75 mm polypropylene tubes (Falcon Labware Division of Becton Dickinson Co., Oxford, CA, USA) containing 0.05 ml of either medium or fMLP in medium, to reach a final concentration of 10 nM. All experiments were carried out in duplicate. The tubes were

incubated at 37°C in a waterbath for 15 min. The incubation was stopped by addition of 0.25 ml ice-cold 10% formaldehyde in 0.05% PBS (pH 7.2). The cell suspensions were kept at 4°C until counting in a hemocytometer using an ordinary light microscope (magnification, 250x). The test was read 'blindly' by two persons; 200 cells were counted from each tube. A cell was 'polarized' if any of the following occurred:

1. elongated or triangular shape
2. broadened lamellipodia
3. membrane ruffling.

The chemotactic responsiveness of a monocyte population was expressed as the percentage of polarized monocytes in the presence of fMLP minus the percentage of polarized monocytes in the absence of fMLP. The percentage of polarized monocytes was calculated as follows:

$$\frac{\%\text{ total cells polarized}}{\%\text{ NSE-positive cells}} \times 100\%$$

Lymphocytes do not exhibit any polarization activity in this assay (18).

Of 77 healthy control individuals tested up till now a mean of 33% polarized monocytes was found (s.d. 11%; ranges from 18 to 70%). There were no differences between female and male individuals: females, a mean of 33%, s.d. 9% (n=36); males, a mean of 32%, s.d. 13 (n=41). Nor were differences found between individuals less than 50 years and over 50 years of age: respectively, a mean of 34%, s.d. 11 (n=66); and a mean of 31%, s.d. 8 (n=11). The inter-assay variation never exceeded 17% (n=13); the intra-assay variation never exceeded 15% (n=77).

Clustering of dendritic cells

The cluster assay as described by Austyn et al. (7) was performed with modifications (17). $5x10^4$ dendritic cells prepared from peripheral blood monocytes exposed to metrizamide were allowed to cluster with $5x10^3$ allogeneic lymphocytes isolated from healthy controls (4 hours, 37°C 5% CO_2) in 250 µl flat-bottomed wells. The lymphocyte isolation was performed according to standard procedures with Ficoll-Isopaque and nylon wool adherence (Leuko-Pak, Fenwall Laboratories, IL, USA). Formed clusters were counted using an inverted microscope and values were expressed as the number of clusters per six microscopic fields (x200). A cluster was defined as an accumulation of 4-25 cells in tri-dimensional configuration.

Serum fractions

Sera were collected from the patients and healthy controls by venipuncture and diluted 1:1 in saline. These dilutions were subjected to ultrafiltration through Amicon CF25 Centriflo cones (Amicon Corp., Danvers, USA) for 15 min at 800 g (molecular weight 'cut off point' 25 kD). The residues were resuspended and stored at -20°C until further use.

Inhibition of monocyte polarization

The capability of the serum fractions to inhibit fMLP-induced polarization of healthy donor monocytes (elutriator purified) was determined by incubating the monocytes ($1x10^6$/ml) for 15 min at 37°C, either with fMLP alone or with fMLP in combination with a serum fraction (final dilution 1:60). The percentage of inhibition was calculated as follows:

$$i = (1 - (Ff\text{-}fo/Mf\text{-}fo)) \times 100\%$$

Ff = % monocytes polarized after incubation with fMLP and LMWF
Mf = % monocytes polarized after incubation with fMLP alone
fo = % spontaneously polarized monocytes

Addition of serum fractions to non-fMLP stimulated donor monocytes did not affect the spontaneous polarization.

Adsorption experiments of LMWF with mAb to p15E

To validate the p15E-like character of LMWF adsorption experiments were carried out by neutralizing the serum fractions, before testing the effects on monocyte polarization, with a combination of two p15E specific mAb (see below) in a final dilution of 1:200 (final IgG concentration 50 μg/ml) at 4°C for 16 h, followed by Amicon ultrafiltration to remove formed complexes: this adsorption/neutralizing procedure was carried out twice (5). The mAb used were a combination of 4F5 and 19F8 (anti-p15E isotypes IgG2a and IgG2b; kindly provided by Dr. G.J. Cianciolo, Genentech Inc., Pharmacological Sciences, South San Francisco, CA, USA). As control antibodies we used irrelevant anti-human IgG2a and IgG2b.

Statistical analysis

Statistical analysis was performed by using the two-tailed Student's t-test, and $p<0.05$ was taken as the level of significance.

RESULTS

Monocyte polarization in HNSCC patients. *In vitro* effects of TP-1

Figure 1 shows the fMLP-induced polarization of monocytes isolated from the peripheral blood of healthy controls and HNSCC patients tested in the absence or presence of TP-1. The fMLP-induced polarization of monocytes from HNSCC patients was lower as compared to the polarization of monocytes from healthy controls: 19% (mean, s.d. 9, range 5-51; n=53) vs 33% (mean, s.d. 7, range 20-49; n=48); significance $p<0.001$. This finding confirmed our previous findings on a defective chemotactic capability of monocytes from patients with HNSCC (3,11).

After the *in vitro* addition of TP-1 to monocytes of HNSCC patients a restoration of the impaired function (viz., the fMLP-induced monocyte polarization) was found (figure 1). A dose-response effect of TP-1 was noticeable (10 patients tested, data not shown). Optimal effects were found at a concentration of 0.1 mg TP-1/ml culture fluid. At none of the concentrations tested (ranging from 0.1 μg to 10 mg TP-1/ml culture fluid) was found any effect of TP-1 on the polarization of healthy donor monocytes. Figure 1 shows the data of the effects exerted by the *in vitro* addition of the optimal dose of TP-1 (0.1 mg/ml) to the monocyte suspensions obtained from a series of 45 HNSCC patients. The original defective fMLP-induced polarization of 19% polarized monocytes (mean, s.d. 9) was improved to the normal value of 33% polarized monocytes (mean, s.d. 11); significance $p<0.0001$. TP-1 had no effect on monocyte polarization in the absence of the chemoattractant fMLP (data not shown).

Clustering capability of DC in HNSCC patients. *In vitro* effects of TP-1

Figure 2 shows the number of DC-lymphocyte clusters obtained after incubating DC prepared from the blood of HNSCC patients or healthy controls with allogeneic lymphocytes. The figure firstly shows that DC prepared from the blood of healthy controls formed 135 (mean) DC-lymphocyte clusters/six microscopic fields (x200). The

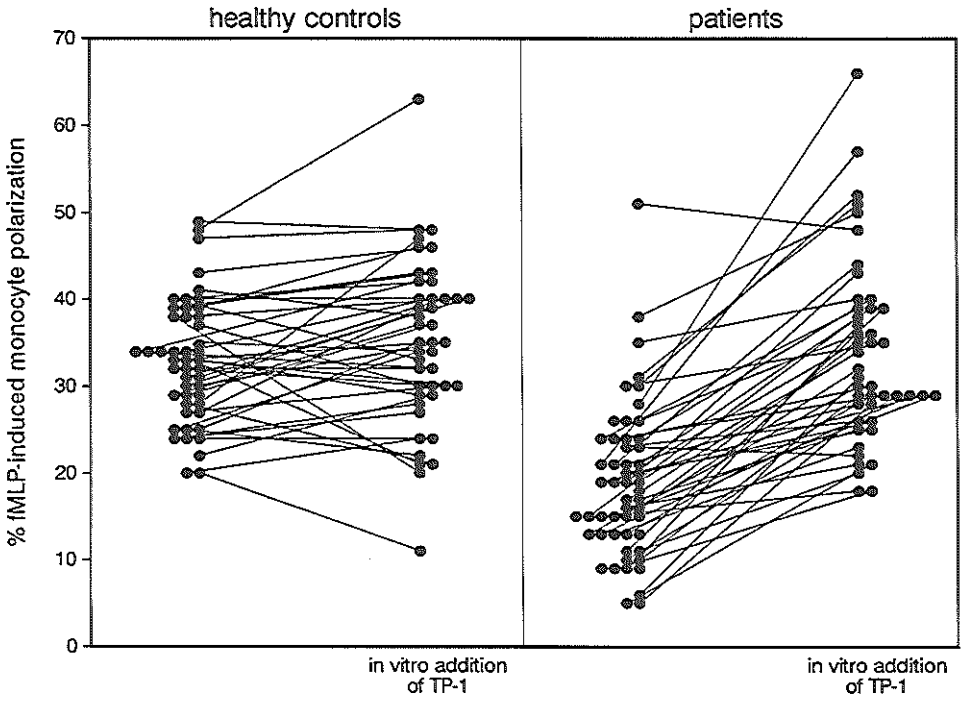

Figure 1. The fMLP-induced polarization of monocytes isolated from the peripheral blood of healthy controls and HNSCC patients in the absence or presence of TP-1 (0.1 mg/ml).

s.d. was 18 (n=39), while outcomes ranged from 90 to 176. On the basis of these data a value of 99 clusters was considered as the lower limit of normality. In 26/44 HNSCC patients the number of formed DC-lymphocyte clusters was defective, and a mean of 94 clusters/six microscopic fields (x200) was found (s.d. 30); significance $p<0.001$.

Secondly, figure 2 shows that the *in vitro* addition of TP-1 also had restoring effects on the impaired capability of HNSCC patient DC to form cellular clusters. In a limited
series of dose-responses 0.1 mg TP-1/ml culture fluid again appeared to be the optimal *in vitro* dosage (5 patients tested; data not shown). The original defective DC clustering of 97 clusters/six microscopic fields (mean, s.d. 36; n=33) was improved to a value of 121 clusters (mean, s.d. 36); significance $p<0.0001$. *In vitro* addition of TP-1 to the DC of healthy controls had no effect.

Inhibition of monocyte polarization by p15E-like factors in the serum of HNSCC patients. Restoring effects of TP-1

The presence of p15E-like factors in the serum of healthy controls and HNSCC patients was determined via their biological effect, namely their suppressive effects on

fMLP-induced polarization of healthy donor monocytes.

Figure 3a shows that the inhibition of fMLP-induced monocyte polarization by LMWF prepared from the serum of patients with HNSCC (III) was 2.6x higher (42%; mean, s.d 13; n=46) than the inhibition obtained with serum LMWF of healthy controls (I) (16%; mean, s.d. 9; n=21); significance <0.001. The suppressive effect of patient serum LMWF was neutralizable with a mixture of two mAb against p15E (4F5 and 19F8): after adsorption of LMWF with the mAb (IV) values of only 19% inhibition (mean, s.d. 9; n=41) were found; significance p<0.001. Control isotype mAb didn't have any effect. A small but statistically significant (p<0.05) neutralizing effect of the anti-p15E was found on the small suppressive effect exerted by LMWF from the healthy controls (I & II).

Figure 3b shows that the *in vitro* addition of TP-1 to the p15E-like serum factors of HNSCC patients (VIII) was capable of neutralizing the suppressive activities of the serum factors on fMLP-induced monocyte polarization (VII) in a similar fashion to the anti-p15E (IV). In dose-response the optimal concentration of TP-1 appeared to range from 67 μg/ml to 6.7 mg/ml, depending on the activity of LMWF used. Since in the limited series of dose-response experiments (6 patients tested; data not shown) 67 μg

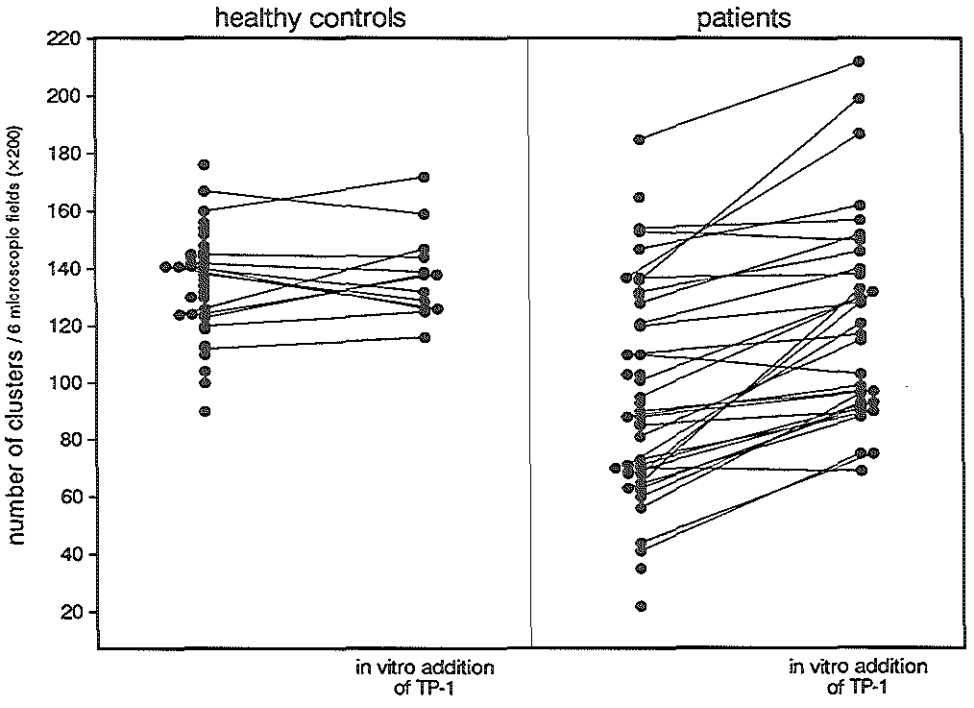

Figure 2. The number of DC-lymphocyte clusters/six microscopic fields (x200) obtained with DC prepared from the blood of healthy controls and HNSCC patients in the absence or presence of TP-1 (0.1 mg/ml).

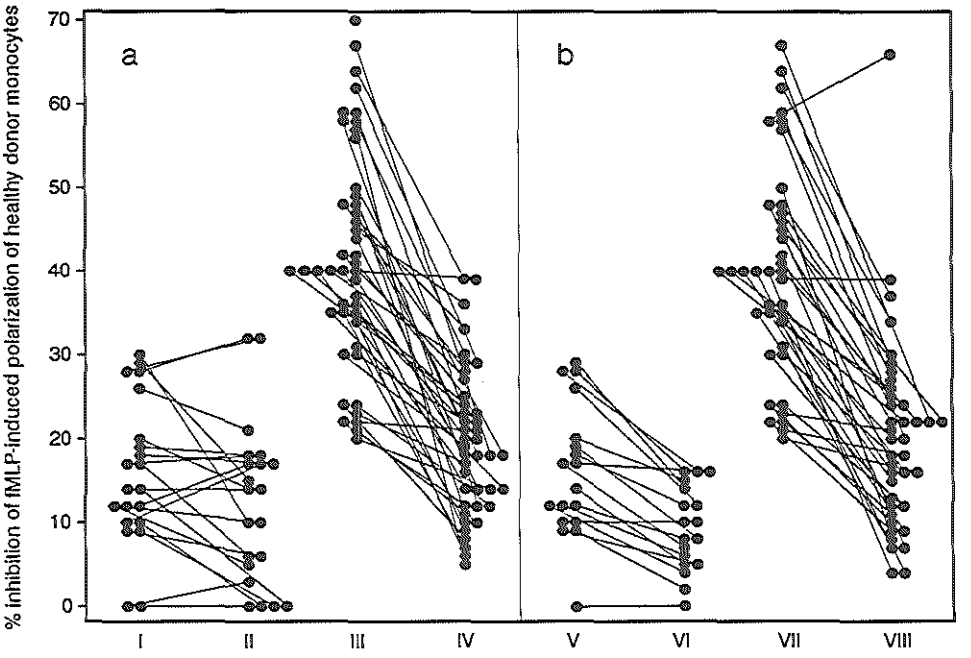

Figure 3. The percentage of inhibition by LMWF prepared from the serum of healthy controls and HNSCC patients on the fMLP-induced polarization of healthy donor monocytes, before or after neutralization with mAb to p15E (anti-p15E) (a) and in the absence or presence of TP-1 (0.067 mg/ml) (b). I, control LMWF: before adsorption with α-p15E; II, control LMWF: after adsorption with α-p15E III, patient LMWF: before adsorption with α-p15E; IV, patient LMWF: after adsorption with α-p15E; V, control LMWF: without TP-1; VI, control LMWF: after addition of TP-1; VII, patient LMWF: without TP-1; VIII, patient LMWF: after addition of TP-1.

TP-1/ml culture fluid always had an effect, we used this concentration in the whole series of experiments. Figure 3b shows that without the addition of TP-1 (VII) a mean of 42% inhibition of monocyte polarization (mean, s.d. 13; n=46) was found, with 67 μg TP-1/ml culture fluid added to the system (VIII) LMWF-induced inhibition was practically restored to normal and values of only 20% inhibition (mean, s.d. 12; n=39) were found (healthy controls: mean 16% inhibition, s.d. 9; n=21); significance $p<0.001$. The addition of TP-1 to healthy donor serum LMWF (VI) had a small but statistically significant effect (without TP-1 mean 16%, s.d. 9; n=21, vs with TP-1 mean 9%, s.d. 5; n=18).

DISCUSSION

In the present study we show a defect in the clustering of DC, matured from the

peripheral blood monocytes of HNSCC patients and confirm our earlier reports on a defective monocyte chemotaxis in HNSCC patients (as measured in the polarization assay) and the presence in patient serum of LMWF related to retroviral p15E capable of suppressing monocyte chemotaxis (3,5,11).

The defect in monocyte chemotaxis in patients with HNSCC has also been confirmed by others (19). These authors additionally found that the number of formylpeptide binding sites on HNSCC patient monocytes was increased despite their defective function. Bugelski et al. (20,21) have reported that in several animal models (the 13762NF MTLn3 rat mammary adenocarcinoma, the B16-BL6 mouse melanoma, the M5076 mouse reticulum cell sarcoma and the autochthonous reticulum cell sarcomas in SJL/J mice) the recruitment of monocytes/macrophages into primary sites or metastases is decreased as tumor mass or the metastases enlarge. This results in a decrease in the density of tumor infiltrated macrophages. Whether our findings of a disturbed polarization of blood monocytes in HNSCC patients also results in a decrease in the density of human infiltrated macrophages and DC into the tumor site is presently being investigated. The defects in clustering of peripheral blood DC found in HNSCC patients reported here are in line with earlier findings of disturbed cell-mediated immune functions, in which DC play a role. There are numerous reports on poor responsiveness to DNCB sensitization in HNSCC patients (22,23), low numbers of peripheral T cells (24,25) and diminished *in vitro* lymphocyte mitogenic responses (23,26). With regard to the presence of DC in HNSCC Nomori et al. (6) showed that patients with a dense infiltration of DC in primary sites survived longer than those without such infiltration. It is noteworthy that there was no such relationship between prognosis and density of lysosome-positive macrophages at the tumor site. This might indicate that the role of DC in tumor defence is more important than the role of tumor-infiltrating macrophages. This view is further supported by the observations of Schroder et al. (27) and Bröcker et al. (28) on the inflammatory cell infiltrates in human papillary thyroid carcinomas and melanomas respectively. In their studies the determination of the quantity of DC at the tumor site also proved to be a highly effective means of assessing the prognosis of such tumors. On the other hand, the degree of infiltration of macrophages and lymphocytes was not associated with a distinct biological behavior. Whether the defective clustering capability of peripheral blood DC in HNSCC patients correlates with a defective infiltration and/or defective function of DC in the tumor itself is also presently being investigated.

Changes in the number and morphology of locally infiltrated DC in response to tumor growth have extensively been studied by Bergfelt et al. (29). These authors showed that the changes found were probably due to a malignant factor released by the tumor which they could not identify. A tumor factor influencing monocyte chemotaxis has been known since 1981. In that year it was demonstrated by Cianciolo et al. (30) that the inhibitory effect on monocyte chemotaxis was due to LMWF produced by both animal and human malignant cells and that this effect could be adsorbed by any of three different mAb to the retroviral capsular protein, p15E. The

presence of these p15E-like factors in HNSCC patients and the suppressive effects on monocyte function have later been described by Balm et al. (5) and Tan et al. (11). The present findings support these earlier reports. It is known that the suppressive effects of p15E-like factors and a 17-amino-acid peptide synthesized from the highly conserved region of MuLV p15E (CKS-17) are not restricted to monocyte chemotaxis, but also have suppressive effects on monocyte mediated killing by inactivating IL-1 (31,32), on the respiratory burst of human monocytes (33), on feline neutrophil activation (34), on the IL-2 or IL-1 dependent proliferation and the blastogenic responses to mitogens and allo-antigens of T cells (35-39) on the human natural killer cell activity of NK cells (40), on polyclonal B cell activation (41) and more relevant to this report, on the clustering capability of DC (42). It is likely that HNSCC-derived p15E-like factors are also responsible for the impaired clustering capability of the HNSCC DC shown in the present report. In a few experiments it was indeed found that the serum p15E-like factor of HNSCC patients suppressed the clustering capability of healthy donor DC (data not shown). With regard to this latter effect, it must be noted, however, that p15E-like factors seem to exert a suppression on the clustering capability of DC which is less marked than the suppression exerted on monocyte polarization (42).

The presence of p15E-like factors in serum is not specific for malignant disease. In Graves' disease (42) and chronic purulent rhinosinusitis (43) the presence of p15E-like factors in serum has also been described. Moreover, p15E-like factors are detectable by immunohistochemical techniques in thymic epithelial cells, epithelial cells overlaying areas of inflammation (44), monocytes (unpublished observations, Tas et al.) and p15E-like factors are produced in macrophages after glucocorticosteroid treatment (45). This suggests that the p15E-like factors are physiological regulators of immune reactivity. Therefore, it is of particular interest that in healthy controls a small activity of LMWF on monocyte polarization was found and that this immunosuppressive activity could partly be adsorbed by the thymic hormone preparation, TP-1, or mAb directed against p15E. In patients with chronic purulent rhinosinusitis treatment with TP-1, resulted in a abrogation of the suppressive effects of the p15E-like serum factor (12). In the present study we showed the *in vitro* restoring effects of TP-1 on defective monocyte polarization and defective clustering of DC. It is likely that TP-1 produced these effects by counteracting the suppressive effects of the p15E-like factors, since in direct competition experiments TP-1 abolished the effects of p15E. Thymic hormones are known to have strong effects on the functioning of the cell-mediated immune system. In neonates they induce a terminal differentiation in T cells, and are able to stimulate T-lymphocytes to produce lymphokines (14,46). In adults the administration of thymic hormones has also been described as effective; and lowered levels of endogenous thymic hormones can be found in several secondary immunodeficiency states, in viral infections and other states of impaired adult host defence (47,48). Beneficial outcomes of thymic hormone treatment in these conditions are generally attributed to a direct effect on T cell maturation. From our data it is however tempting to speculate that other mechanisms may be involved as well and that these thymic factors may exert, at least in part, their effect via cells of the monocyte-macrophage-DC

system and via the neutralization of an endogenously produced immunosuppressive factor. Such an effect of thymic hormones could explain their beneficial effects in adults with malignant growth and cell-mediated immunodeficiencies in the presence of a full T cell repertoire (49).

The observations reported here on the *in vitro* effects of TP-1 on depressed monocyte and DC function in HNSCC has provided one of the rationales for a recently started TP-1 therapeutical pilot trial in HNSCC patients.

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8

DISCUSSION

This thesis describes:

a) the presence of a gp41-like monocyte polarization inhibiting factor in the serum of human immunodeficiency virus (HIV)-infected men;
b) the presence of a p15E-like monocyte polarization inhibiting factor in the serum of:
   - patients suffering from Graves' disease
   - patients suffering from defects in cell-mediated immunity (CMI) and chronic purulent rhinosinusitis (CPR)
   - patients suffering from head and neck squamous cell carcinoma (HNSCC)
c) defects in monocyte polarization in the patient groups described in b) which were accompanied by defects in dendritic cell clustering, both defects being related to the presence of p15E-like factors in the serum;
d) the restoring effects of the partially purified thymic hormone preparation TP-1 on the immunosuppression exerted by the p15E-like serum factors, both *in vitro* and *in vivo*.

8.1 Amino acid sequences of p15E and gp41 in relation to their immune reactivity

CKS-17 homologues and immunosuppression

In p15E of the murine and feline leukemogenic retroviruses a high degree of homology within a 26 amino acid (aa) region has been noted (1-3). The synthetic peptide CKS-17 (4), consisting of the first 17 aa of the most closely conserved region of p15E, has many p15E-like immunosuppressive effects (see Introduction, table 2). A constructed peptide corresponding to a similar conserved sequence of the transmembrane envelope protein (TM-protein) HIV gp41 shows comparable immunosuppressive effects (5-7). The aa sequences of this region and the percentage identity with CKS-17 are listed in table 1.

Since CKS-17 is able to suppress monocyte polarization (15), it is tempting to speculate that the monocyte polarization inhibiting factor described in chapter 2 is due to the effect of the CKS-17-like stretch in HIV gp41. However, it must be noted that there is only a limited sequence homology between the HIV sequence and CKS-17 (HIV-1, 37%; HIV-2, 32%). One explanation may be that the tertiary conformation of both peptides is a critical factor in their activity and that they share similar three-dimensional structures. Such an explanation requires further investigation. In support of such a view it can be noted that relaxin and insulin have almost identical three dimensional structures yet share less than 25% aa sequence homology (16). Moreover, in chapter 2 we have shown that the monoclonal antibody (mAb) to gp41 and the mAb to p15E were both able to adsorb the immunosuppressive effects of the HIV lysate, the recombinant gp41 and some serum fractions of HIV infected men. This suggests that both mAb reacted with similar conformational epitopes in gp41 and in p15E.

The aa sequences of conserved regions of retroviruses, other than HIV, in relation to CKS-17 are also listed in table 1. Cianciolo et al. (4) demonstrated that the HTLV-I

Table 1. (According to Oostendorp et al., 15) CKS-17, a synthetic heptadecapeptide derived from a conserved region of the TM-protein of MuLV and CKS-17 homologues derived from the aa sequences of the TM-proteins FeLV, BLV, MPMV, HTLV-I, HTLV-II, HIV-1, HIV-2 and the endogenous sequences of corresponding regions in SMRV-H, ERV-3 and ERV-4.1, and CS-3, the synthetic peptide derived from HIV-1 gp41.

species	virus	aa sequence	refs	% identity to CKS-17	% identity to LDLLFL
	CKS-17	LQNRRG LDLLFL KEGGL	4		
murine	MuLVs	LQNRRG LDLLFL KEGGL	1	100	100
feline	FeLV	LQNRRG LDILFL KEGGL	1	94	83
primate	MPMV	LQNRRG LDLLTA EQGGL	8	86	67
human	HTLV-1	AQNRRG LDLLFW EQGGL	1,9	86	83
human	HTLV-2	AQNRRG LDLLFW EQGGL	1,9	86	83
human	SMRV-H	LQNRRG LDLLTA EQGGL	10	86	67
bovine	BLV	LQNRRG LDWLYI RLGSL	1	65	50
human	ERV-3	YQNRLA LDYLLA QEEGL	11	49	50
human	ERV-4.1	YQNRLA LDYLLA AEGGV	12	49	50
human	HIV-1	LQARIL AVERYL KDQQL	13	37	17
human	HIV-2	LQARVT AIEKYL KDQAQ	14	32	17
	CS-3	LQARIL AVERYL KDQQL	5	37	17

One letter codes represent L-amino-acids: A=alanine, D=aspartic acid, E=glutamic acid, F=phenylalanine, G=glycine, I=isoleucine, K=lysine, L=leucine, P=proline, N=asparagine, Q=glutamine, R=arginine, S=serine, T=threonine, W=tryptophan and Y=tyrosine. The % identity to the CKS-17-derived hexapeptide LDLLFL is given.

sequence has similar immunosuppressive capabilities to CKS-17. The endogenous human retroviral sequence of ERV-3 showed a slight but statistically significant inhibition of anti-CD3-stimulated lymphocyte proliferation. The authors explained this finding by supposing that only a subpopulation of cells is sensitive to the effect of this endogenous human retroviral sequence.

With regard to CKS-17 itself, Oostendorp et al. (15) have found that of the various hexapeptides which they constructed from the CKS-17 domain, the LDLLFL sequence suppressed N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced polarization of monocytes and granulocytes. Several hexapeptides constructed as homologues to the CKS-17 domain of the retroviral TM-proteins, and corresponding to the LDLLFL sequence (underlined, table 1), also had an inhibiting effect on monocyte and

neutrophil polarization, demonstrating that this region of 6 aa may play a crucial role in retrovirus-associated immunosuppression. It is important to note, however, that practically no homology exists between the CKS-17 hexapeptide LDLLFL and the corresponding HIV-1 hexapeptide AVERYL. The inhibition of monocyte polarization by the latter sequence was less clear, suggesting that this region of 6 aa of the HIV-1 peptide is not sufficient for the suppressive effect exerted by the 17 aa sequence.

Monocytes appeared to be more sensitive to the suppressive activity of the CKS-17 hexapeptide LDLLFL than lymphocytes or NK cells (15). The mechanisms of action of this hexapeptide and the interactions with receptors therefore need further investigation. It is likely that other sequences are required for the full immunosuppressive effects of p15E, gp41 and related retroviral peptides.

Mechanisms of action

With regard to the mechanism of action of CKS-17, a novel mechanism of inhibition of protein kinase C (PKC) activity has recently been reported (17). CKS-17 inhibited PKC-catalyzed Ca^{2+}- and phosphatidylserine-dependent histone phosphorylation, but did not inhibit the catalytic subunit of cAMP-dependent protein kinase. CKS-17 also inhibited the Ca^{2+}- and phosphatidylserine-independent activity of a catalytic fragment of PKC that was generated by limited trypsin treatment, but did not act as a competitive inhibitor of PKC with respect to ATP or phosphoacceptor substrates and pseudosubstrates of PKC (17).

With regard to the HIV-1 CKS-17 homologue LQARILAVERYLKDQQL (aa581-597), a similar mechanism of action has been described by Ruegg & Strand (7). They showed that this peptide from the HIV-1 TM-protein gp41 inhibited PKC-mediated phosphorylation of the CD3 γ-chain in intact cells and directly inhibited partially purified PKC. The inhibition was non-competitive with respect to the substrates histone and ATP and independent of the regulatory domain of the enzyme. Additionally, they showed that this peptide specifically inhibited the intracellular Ca^{2+} influx in Jurkat cells stimulated by the mAb OKT3, whereas it had no effect on the production of inositol triphosphate. Furthermore, they demonstrated that the peptide required internalization for inhibitory activity (7).

In conclusion, despite the very limited sequence homology between the MuLV p15E and HIV-1 peptide gp41, the proteins share similar immunosuppressive properties. It is likely that the proteins share conformational epitopes that are responsible for the immunosuppressive activities. It is also likely that the CKS-17 region in both proteins is part of this conformational epitope, since peptides constructed to this region share similar immunosuppressive properties and mechanisms of action.

TM-protein immunodominant regions as targets for B and T cell responses

The structural features of the immunodominant region in gp41 which contribute to its activity have not yet been fully characterized. The region is relatively hydrophobic, and was not identified as strongly immunogenic by computer-assisted analysis (18).

However, the immunosuppressive regions of retroviral TM-proteins not only exert effects on cells of the immune system, but are also the targets for B and T cell responses. This has been shown in detail for HIV gp41.

With regard to the epitopes for B cells, serodiagnostic assays have demonstrated that HIV-1 gp41 is the antigen most consistently recognized by antibodies in sera from patients with AIDS and AIDS-related complex (19). Although disputed (20), several reports suggest that the antibody response to gp41 is mainly directed toward an immunodominant region, which is broadly defined between aa580-620, having the sequence LQARILAVERYLKDQQLLGIWGCSGKLICTTAVPWNAS (21-27; table 2). The above described immunosuppressive CKS-17 homologue of HIV-1, the CS-3 peptide LQARILAVERYLKDQQL (aa583-599, Cianciolo et al., 5; aa581-597, Ruegg and Strand, 6) is within this region (note that the literature is not consistent with respect to the position number of the amino acids, because of the use of sequences from different HIV-isolates; table 2).

Not only have antibody responses to various gp41 regions been studied, but also T cell responses. Schrier et al. (28) have demonstrated that 7 of 29 HIV-1 seropositive but 0 of 13 HIV-1 seronegative donors exhibited T helper cell responses to the gp41 aa584-609 peptide and Hammond et al. (29) have reported that this region is a conserved T cell epitope in HIV-1 gp41 recognized by vaccine-induced human cytolytic cells.

The B cell and T cell epitopes in viral p15E have not yet been defined. Nevertheless Thiel et al. (30) have suggested, using an experimental model of an AKR murine leukemia, that α-p15E antibodies play a role in preventing the immunosuppressive effects of p15E.

This hypothesis has again been extended to HIV-1, for which it was reported that antibody reactivity with the HIV-1 gp41 aa583-599 peptide (pHIVIS) was strongly associated with the absence of HIV-related immunosuppression (21). However, recently Cheingsong-Popov et al. (31) reported that antibodies to pHIVIS did not correlate significantly with the outcome of the HIV infection. Moreover, it has been reported that two human mAb directed against HIV-1 enhanced HIV-infection *in vitro* (32) and that both bound to the aa586-620 region of gp41 (33). The *in vivo* relevance of this complement-mediated, antibody-dependent enhancement of HIV-1 infection has still to be determined.

Immunodominant regions and actions of retroviruses

It is interesting to note that the immunodominant regions of TM-proteins may also play a role in retrovirus-mediated cell lysis, infection and release of virus from infected cells. Qureshi et al. (34) demonstrated that incubation of RH9 cells with CS-3 prior to addition of HIV-1 prevented HIV-1-mediated cell lysis and inhibited infection (table 2). Wegemer et al. (35) showed that a synthetic peptide, consisting of the CKS-17 region synthetically linked to another short TM-derived sequence, blocked in a nontoxic and sequence-specific manner the release of MuLV from two chronically infected cell lines.

Table 2. The so called immunodominant region of the TM-protein gp41 (A) and other regions of gp41 (B) in relation to immune reactivity.

A	LQARILAVERYLKDQQLLGIWGCSGKLICTTAVPWNAS		Immunodominant region
	598 — 609*	Gnann et al.[1]	
	603 — 609	Gnann et al.[2]	
	586 — 606	Wang et al.[3]	
	599 — 613*	Narvanen et al.[4]	
	583 — 599	Cianciolo et al.[5]	CS-3
	581 — 597	Ruegg & Strand[6]	
	584 — 609	Schrier et al.[7]	
	584 — 595	Hammoud et al.[8]	
	583 — 599	Klasse et al.[9]	pHIVIS
	583 — 599	Chelngsong-Popov et al.[10]	pHIVIS
	586 — 620	Robinson et al.[11]	Primary immunodominant region
	583 — 599	Qureshi et al.[12]	CS-3
	(LQNRRGLDLLFLKEGGL)**	Wegemer et al.[13]	CKS-17
B	735-YDRPEGIEEEGGERDRSGC-752	Chanh et al.[14]	
	846-CAYAIRHIPRRIRQGLERG-860	Chanh et al.[15]	
	850-LERLLL-855	Reiher et al.[16]	
	(2-LEHLLL-6)**	Reiher et al.	IL-2A
	(LDLLFL)**	Oostendorp et al.	CKS-17-hexapeptide (+ one aa)
	841-RHIPRRIRQGLERILL-856	Weigent et al.[17]	
	524-LFLGFLGAAGST-535	Gallaher/Rafalski et al.[18]	
	(LDLLFL)**	Oostendorp et al.	CKS-17-hexapeptide
	838-EGTDRVI-843 Golding et al.[19]		
	(20-NGTERVR-24)**	Golding et al.	β1 domain of HLA class II β chain

* position numbers of aa sequences of different HIV isolates; ** sequence identity of other regions with gp41 regions (see text); 1-4,9,10 epitope for neutralizing antibody response; 5,6 immunosuppression; 7 epitope for T helper response; 8 epitope for T cytotoxic response; 11 epitope for HIV-infection enhancing antibody response; 12,13 interference with infection; 14,15 immunosuppression; 16 IL-2 antagonist activity; 17 no antagonist or immuno-suppressive activity; 18 fusion peptide; 19 epitope for inhibitory antibody response by mimicking MHC class II.

Other TM-protein regions and immunosuppression

With regard to immunosuppression, sequences other than the immunodominant region of HIV-1 gp41, viz., the sequences aa735-752 and aa846-860, have also been shown to have immunosuppressive properties (36,37; table 2). The aa846-860 peptide (LERLLL) contains a six-residue region of sequence identity with the LDLLFL hexapeptide of CKS-17 and with IL-2 (38,39). The immunosuppressive activity of this peptide (38) was explained as an IL-2 antagonist activity. However, a synthetic hexadecapeptide containing this hexapeptide and the adjacent decapeptide (HTLV-III/BH10 residues 841-856) exhibited none of these immunosuppressive activities (39). Regarding the aa sequence homology of HIV-1 gp41 with the CKS-17-derived LDLLFL sequence, this sequence also appears to be highly homologous to the N-terminal region of gp41 (LFLGFL; table 2), which region is likely to be involved in infection as a fusion peptide (40). Rafalski et al. (41) demonstrated that peptides representing the N-terminal 23 residues of gp41 can adopt helical structures and interact strongly enough with phospholipids to disrupt bilayer membranes, although the exact significance of this in biologically relevant fusion events remains to be established.

Another sequence of gp41 is homologous to a conserved five aa sequence in class II MHC molecules (42; table 2). About one third of HIV-infected individuals (regardless of disease stage) produce antibodies reactive with peptides derived from this region of gp41. These sera also react with class II MHC molecules and inhibit the proliferation of normal $CD4^+$ T cells to tetanus toxoid or allogeneic cells (43). This inhibitory activity was largely removed by adsorption with sepharose-linked class II peptides or gp41 peptides (43). Thus, whereas antibodies to the putative immunosuppressive domain of gp41 may protect against immunosuppression, antibodies to the class II MHC-homologous sequence of gp41 may cause immunosuppression.

In conclusion, HIV gp41 and the antibody reaction towards this protein are capable of inducing complicated forms of immunomodulation, either of an inhibiting or of a stimulating character. It is likely that p15E exerts similar complex reactions on the immune system.

Immunologic effects of other structural retroviral proteins

This thesis emphasizes on the immunosuppressive effects of these retroviral TM-proteins and their related peptides. However, it must also be noted that other structural proteins of retroviruses have immunologic effects. Recently, a murine retrovirus-induced immunodeficiency syndrome has been shown to result from a defective retrovirus which encoded an altered gag protein (44,45). This altered gag protein appeared to be responsible for the lymphoproliferation and immunodeficiency that developed in this syndrome. Moreover, some retroviruses encode additional proteins beside gag, pol and env. These proteins often regulate viral transcription, but can also affect host transcription. For example, the HTLV-I trans-activating protein, tax, has been shown to increase expression of the cellular genes for IL-2, IL-2-R, GM-CSF and IL-3 (46-48).

Such activation could dramatically alter the growth of infected cells, and could have secondary effects on uninfected lymphoid cells.

8.2 The presence of p15E-like factors in the serum of patients with disturbances of the immune system

Figure 1 of this Discussion shows the p15E-related activity in the serum of all patients who have been tested in our laboratory during the last few years. They include 79 healthy controls, 41 patients with squamous cell carcinoma of the head and neck, 16 patients with CPR and defects in monocyte polarization, 24 patients with Graves' disease, 17 patients with type 1 insulin-dependent diabetes mellitus (IDDM) and 31 patients with Sjögren's syndrome (SS).

The biological activity of the p15E-like factors in the serum of these patients is expressed as the inhibition of fMLP-induced polarization of healthy donor monocytes by the serum fraction <25kD minus the inhibition of the serum fraction after adsorption with α-p15E mAb (for details on the techniques, see chapter 3).

The highest activity of serum p15E was found in patients with defects in monocyte polarization and concomitant CPR (all patients positive with a mean of 54% inhibition of monocyte polarization, s.d. 17) and in patients with Graves' disease (20 out of 24 patients positive with a mean of 31% inhibition, s.d. 16). In the other immune diseases tested p15E-like factors were present at moderate biological activity. In IDDM 11 out of 17 patients were positive with a mean of 19% inhibition (s.d. 10); in SS 10 out of 31 patients were positive with a mean of 12% inhibition (s.d. 8). Interestingly, the serum of our SS patients positive for antinuclear antibodies (ANA), anti-Ro (SS-A) and anti-La (SS-B), were all positive for p15E.

Although 61% of patients with head and neck squamous cell carcinoma were positive (25 out of 41 patients), p15E-like factors were present at generally moderate biological activity with a mean of 23% inhibition (s.d. 10).

The origin of the serum p15E-like factors in above described patients is speculative. Putative possibilities (see Introduction and the various chapters) are:

a) an exogenous infection with an as yet unknown retrovirus possessing envelope proteins that share structural homology with murine and feline p15E;
b) an aberrant autonomous production of endogenous-encoded factors by derailed cells; and
c) an enhanced physiological production of endogenous-encoded factors by monocytes or epithelial cells in response to immune reactivity.

p15E-like factors in malignant disease

The presence of p15E-like factors in the serum of patients with squamous cell carcinoma of the head and neck is most probably due to an aberrant autonomous production of the factors by the derailed tumor cells, since p15E-like factors disappear after successful surgical removal of the tumor (this thesis, chapter 5). The continuous

presence of the factor can be related to residual or recurrent disease. Other authors have also reported that surgical removal of the tumor restored defective immune functions related to p15E (49,50).

The aberrant production of p15E-like factors by tumor cells is most probably due to an abnormal expression of endogenous retroviral-like sequences. A retrovirus infection of the tumor cells is less likely, since it has been reported that several types of tumor cell lines express p15E-related molecules without being infected with retroviruses (51).

Furthermore, it has been established that not only head and neck cancer cells express p15E-related molecules. Therefore the presence of p15E-like factors in serum is not exclusive to patients with squamous cell carcinoma of the head and neck. In preliminary experiments at our laboratory, sera from 21 out of 30 patients with a malignant tumor of the breast showed p15E-like activity (Stöger et al., manuscript in preparation). Sera from 29 out of 30 patients with a benign adenoma of the breast did not. This suggests that only malignant neoplastic cells produce p15E-like factors. It can even be speculated that the expression of p15E-like factors is necessary to obtain the malignant state. In this respect it is interesting that the GLDLLFL sequence of CKS-17 is 60% identical to a 7 aa sequence in v-src (GSLLDFL, 52), which is a transducer of growth factor response (a peripheral membrane protein-tyrosin kinase), and v-erb-B

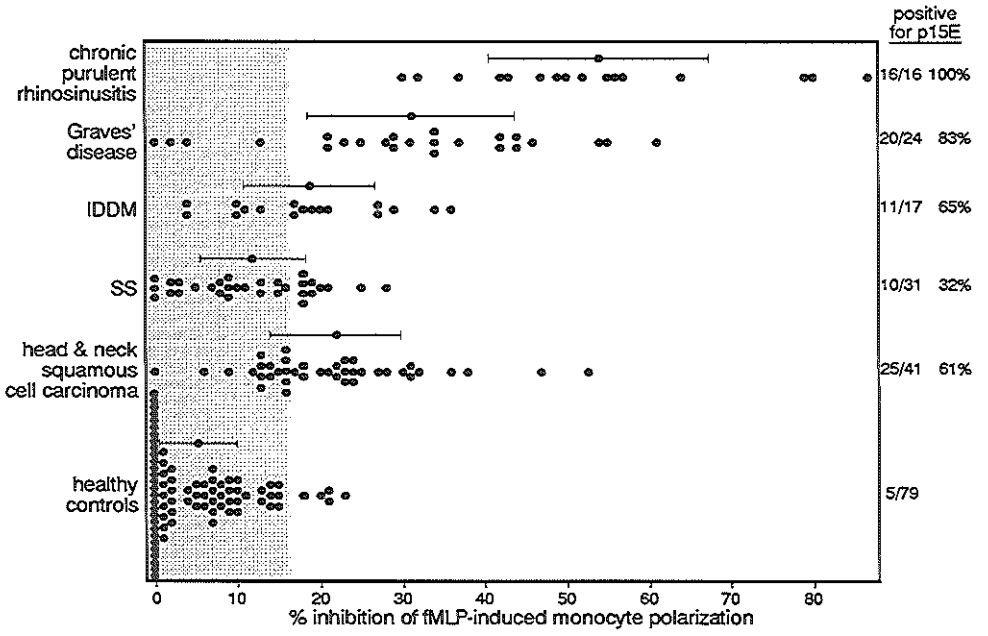

Figure 1. p15E-related activity in sera from healthy controls and patients with HNSCC, SS, IDDM, Graves' disease and CPR.

(GCLLDYL, 53), which is a truncated form of the receptor for epidermal growth factor (an integral membrane protein-tyrosin kinase). V-src and v-erb-B belong to two different classes of the so-called oncogenes (54). Oncogenes were first described as retrovirus-encoded genes that produced tumors in birds and rodents. These genes were then shown to be dominant mutants of host genes (proto-oncogenes) that had been picked up by the retroviruses (55,56). Most proto-oncogenes are thought to encode proteins that are involved in the cascade of events by which growth factors stimulate normal cell division. Whether p15E-like factors are also involved in the growth responses of tumor cells needs further investigation.

p15E-like factors in autoimmune diseases

Although it is clear that infectious and endogenous retroviruses can have profound effects on the functioning of the immune system, it is not yet clear whether infectious and/or endogenous retroviruses are involved in the pathogenesis of autoimmune disease (57). A putative retroviral involvement has been elegantly demonstrated in SS.

SS is an autoimmune disease which shows some signs and symptoms in common with the syndrome induced by HIV infection (58,59). The dryness of the mouth and eyes in both SS and HIV disease is due to loss of salivary and lacrimal gland function and is accompanied by lymphocytic infiltration of these glands (59). An additional link between SS and HIV is the observation of Talal et al. (60) that approximately 30% of primary SS patients produce serum antibodies that react with the major capsid protein of HIV (CA, p24/25). In some SS patients, reactivity to another gag protein, p17 (MA), was also observed. Similar percentages of patients with systemic lupus erythematosus (SLE), scleroderma, and juvenile rheumatoid arthritis (JRA) also produce HIV gag-reactive serum antibodies (60,61), as well as individuals with other chronic diseases and some healthy individuals (1-4%). These observations and the isolation of retroviral particles from cells incubated with salivary gland homogenates of patients with SS (62) suggest the possibility of a retroviral etiology in SS. Interestingly, some lines of mice transgenic for the retroviral tax protein develop an apparently autoimmune exocrinopathy similar to SS, suggesting a potential for the HTLV-I tax gene to induce autoimmunity (63).

Our finding of the presence of p15E-like factors in about 30% of patients with SS (figure 1) could be taken as a further support of a connection of SS with retroviral infection. However, the possibility that p15E-like factors are endogenously produced as the consequence of an excessive immune reactivity is still another plausible possibility. It remains to be clarified whether the serum of SS patients positive for p15E also contains antibodies that react with p24/25 of HIV.

As mentioned before, the serum of our SS patients positive for ANA, SS-A and SS-B antibodies, were all positive for p15E. Interestingly, a major auto-epitope of SS-B/La shares amino acid homology (up to 6 out of 8 aa identical) with CA proteins from several retroviruses, including HIV (64). Such homology raises the intriguing possibility that anti SS-B/La autoantibody production could result from an immune response to

homologous endogenous or exogenous retroviral proteins.

With regard to IDDM, we also found some serum fractions to contain p15E-like factors (figure 1). Current evidence indicates (65) that IDDM is a progressive autoimmune disease resulting in complete beta cell destruction in genetically susceptible individuals. Histocompatibility genes are major determinants of this genetic susceptibility and more than 90% of IDDM individuals express the HLA alleles DR3 or DR4. Prior to clinical diabetes islet-cell antibodies and activated T lymphocytes are found in conjunction with a slowly progressive loss of intravenous glucose stimulated insulin secretion (66).

A number of viruses has been shown to induce diabetes in animal models (67). Persisting IgM responses to Coxsackie virus, as found in some diabetic children (68,69), may either indicate a defect in the switching to an IgG specific response or may indicate that these individuals clear the viral antigen less efficiently (70). In addition, T cell hyporesponsiveness to mumps and Coxsackie B4 viruses has been found in DR3 positive subjects in general (71). Another possibility is that slow viruses might be implicated in the development of diabetes. Impaired function of morphologically normal beta cells has been demonstrated following infection of mice by lymphocytic choriomeningitis virus (72). Whether the retrovirus-related p15E-like factors reflect a retroviral infection in IDDM or reflect an endogenously encoded response to immune reactivity still has to be determined. Interestingly, Leiter et al. (73) reported that in a mouse model of diabetes, hyperglycemia induced the expression of endogenous type A retroviral particles in beta cells, which might contribute to disease development.

The highest levels of p15E-like factors in autoimmune disease were found in Graves' disease (figure 1). Graves' disease is now clearly established as an organ specific autoimmune disease due to thyroid stimulating hormone receptor (TSH-R) antibodies occurring in patients with a genetic susceptibility for immune dysregulation (74). This, however, does not rule out an involvement of viruses in the onset of this autoimmune disease as well as in other thyroid autoimmune phenomena.

Mild thyroiditis and thyroid antibody production were previously observed after infection with reovirus Type I in BALB/c mice and, recently, the segment of the specific reoviral genome for thyroid tropism was identified, i.e. the S1 segment coding for the sigma 1 hemagglutinin polypeptide (75). Infected mice produced antibodies to the second colloid antigen and to thyroglobulin. In this context, it is worthy to be mentioned that both mothers and children with sporadic congenital hypothyroidism also show antibodies to the second colloid antigen, supporting the putative role of a viral infection in this disease (76).

Few reported attempts are described to isolate retroviruses from patients with thyroiditis. Stanćek et al. cultured a novel virus from five of eight Czechoslovakian patients with de Quervain subacute thyroiditis (77). Serologic studies suggested that 20 out of 28 patients were 'highly' or 'moderately' positive for neutralizing antibodies to this virus, while only one of 15 controls had 'moderate' neutralizing antibodies. This virus was subsequently identified as a human foamy retrovirus (78,79). However, the

significance of foamy virus infection in the field of thyroid autoimmunity remains uncertain.

The involvement of endogenous retroviral sequences in the initiation of Graves' disease was highlighted by the recent findings of retrovirus-like HIV-1 gag sequences by Southern blotting of DNA extracted from the thyroid and lymphocytes of Graves' patients (80). These data have, however, not been subsequently confirmed by other groups (81). Ongoing investigations have shifted attention to HTLV-1 in Graves' disease, and positive signals have been identified in DNA of peripheral blood mononuclear cells of these patients with or without IDDM (82). Cloning and sequencing of the relevant polymerase chain reaction (PCR) products so far identified should sustain the bona fide of the initial hypothesis that has proposed retroviruses as putative triggering agents in the mediation of autoimmune thyroid disease. With respect to the endogenous retroviral sequence (ev22) found in the genome of the obese strain chickens, it is now believed that its presence is closely linked to a disturbed pituitary-adrenal axis response to IL-1 in these animals (83).

It should also be taken into consideration that the p15E-like factor found in the sera of patients with IDDM and Graves' disease may be the effect of a physiological response to immunoreactivity, and this may be more likely than a viral involvement. Arguments are given in chapter 3, where it is described that normal thyrocytes contain p15E-like material, and a physiological role of endogenous p15E is suggested.

In conclusion, the origin and the role of p15E-like factors in autoimmune diseases, such as SS, IDDM and Graves' disease, is not clear yet. These factors can be endogenously produced to regulate the immune response or can be present in serum as the consequence of infection with an as yet unknown retrovirus.

p15E-like factors present in patients with defects in CMI and CPR

About 60% of patients suffering from unexplained relapsing CPR had defects in CMI (chapter 4). All patients with a defective monocyte polarization were positive for p15E-like factors (figure 1).

In contrast to the autoimmune condition of e.g. Graves' disease, the origin of the p15E-like factors is more likely to be viral. Arguments are firstly that the bioactivity of p15E-like factors in this patient group is higher than in all the other patient groups tested and secondly that the p15E-related bioactivity is comparable with the bioactivity of gp41-like factors found in HIV-seropositive patients. Experiments of Scheeren et al. (manuscript submitted for publication) have shown p15E positivity of nasal mucosa epithelial cells in nasal polyposis. The nasal mucosa may be a portal of entry for this putative virus, resulting in the consequent production of the p15E-like factors found in the serum. It should be noted that several of our patients with CPR clearly indicated a 'flu-like syndrome' just prior to the onset of their chronic upper respiratory tract complaints. It is possible that the putative virus (like many other retroviruses) possesses a tropism for lymphoid cells. Further research should unravel whether the lymphoid cells in the nasal mucosa of our patients with defects in CMI and CPR harbour yet unknown retroviruses.

An important observation is that some of our patients with SS show relapsing airway infection and that some of our patients with CPR were characterized by a lymphocytic infiltration of the salivary and lacrimal glands. Both observations indicate the possibility that in a proportion of the patients the syndrome of CMI defects and CPR is a variant of SS. As in SS this would mean that the p15E-like factors detected in the serum of patients with CMI and chronic purulent infections of the upper respiratory tract reflect either a retroviral infection or an endogenous production of the regulating factor as a consequence of an excessive immune reactivity.

8.3 A role for p15E-like factors in the regulation of immune homeostasis

Let us now concentrate on p15E-like factors as normal physiological immune regulatory factors. It should be noted that the inhibition of monocyte polarization due to p15E-like factors almost never exceeds a 15% level in healthy controls (figure 1). However, a small but statistically significant neutralizing effect of α-p15E mAb was found on the small suppressive effect exerted by the serum fractions <25kD of the healthy controls, indicating a low level of p15E-related bioactivity in their serum.

The expression of p15E-like material in normal tissue (e.g. in monocytes and epithelial cells of the thymus, see introduction) strongly suggests that the p15E-like factors are physiological regulators of immune reactivity and therefore it is not surprising that p15E-like factors are present in moderate quantities in the serum of healthy controls.

If endogenously produced p15E-like factors play a role in the regulation of immune homeostasis, these factors may be considered as cytokines. Some of our recent findings (Simons et al.; preliminary data) point in the direction that interferon alpha (IFN-α) and p15E are very much related.

Firstly, similarities exist between CKS-17 and a conserved region present in virtually all IFN-α sequences of both human and animal origin (84); secondly, some of the biological effects of retroviral TM-proteins are shared by IFN-α, including inhibition of lymphocyte proliferation (85) and the inhibition of polarization of healthy donor monocytes (however, very high concentrations of IFN-α are needed to obtain similar effects as CKS-17; preliminary data); thirdly, immunohistochemical studies performed in our laboratory show that head and neck tumor cells stain positive using a mAb or polyclonal antibody to IFN-α; and fourthly, a mAb to IFN-α was able to neutralize the effect on monocyte polarization induced by the p15E-like factors from the serum of patients with head and neck squamous cell carcinoma as described for the mAb directed against p15E (chapter 5 and 7).

These data suggest that p15E-like factors are related to the IFN-α family, because they clearly share epitopes as well as biological activity.

Transforming growth factor beta (TGF-β) is another cytokine that is partially homologous to CKS-17 and that shares biological effects with this peptide (86) as well as IFN-α. Therefore, the relation between p15E-like factors, IFN-α and TGF-β needs

further exploration. The exact identification of functionally active domains in these cytokines and the construction of biologically active synthetic peptides from these domains is required to further define potentially shared mechanisms of action between p15E-like molecules and these cytokines. Another approach might be a study at receptor level. Since a specific association of retroviral p15E with human cell surfaces has recently been described (87), interaction of p15E with cytokine receptors of cells responsive for IFN-α or TGF-β could be examined.

The above described data suggest that viral p15E may have evolved from or has utilized genetic codes of the active sites of IFN-α, TGF-β and possibly other cytokines because of the selective advantage gained from having such activities. However, the limited degree of similarity may simply have arisen by random mutation and selection of mutants having desirable structural properties. On the other hand, reintegrated endogenous retroviral sequences may play a role in physiological immunoregulatory systems as p15E-like members of the family of cytokines.

A role for endogenous retroviral p15E-like sequences in the regulation of lymphocyte activation in mice is described in the Introduction of this thesis. That p15E-related factors may play a role in normal immune homeostasis in mice is nicely illustrated by the recent work of Helmberg et al. (88). These authors reported increased mRNA steady-state levels transcribed from an endogenous ecotropic type C retroviral locus in glucocorticoid-treated macrophages giving rise to the transmembrane retroviral protein p15E. The authors suggested that the immunosuppressive activity of glucocorticoids could therefore be mediated, at least in part, by regulating the expression and production of p15E-like factors in monocytes/macrophages.

Glucocorticoids can also mediate immunosuppression by selectively inhibiting the transcription of the IL-1-β gene and destabilizing the mRNA for IL-1-β (89). Thus, it is of particular interest that Kleinerman et al. (90) reported that the CKS-17 sequence of retroviral p15E blocked the direct cytocidal activity of IL-1-β against A375 tumor cells. In addition, glucocorticoids inhibit the accumulation of interferon gamma (IFN-γ) mRNA in PHA-stimulated lymphocytes (91); IFN-γ production of human peripheral mononuclear cells has also been reported to be inhibited by CKS-17 (92). However, the effects of glucocorticoids involve such a variety of action (93) that a connection with p15E seems speculative.

The ideas described above suggest a role of p15E-like factors as cytokines in physiological immunoregulation.

8.4 Thymic factors or hormones are capable of abrogating the effects of p15E-like factors

The thymic hormones were the first immunomodulating hormones in clinical use, and they have been reported to induce raised numbers of circulating T-lymphocytes in immunologically impaired patients, as well as improvements in lymphocyte blastogenic responsiveness to mitogens and improvements of delayed hypersensitivity

skin responses (94-97).

Although literature on thymic hormones usually focuses on these T cell improvements, our studies (chapters 6 and 7) also show a clear effect of the partially purified thymic hormone preparation TP-1 on the functioning of monocytes and dendritic cells. Since TP-1 consists of various peptides including the defined molecules thymosin-α1 and thymopoietin, we considered it important to examine the neutralizing activity of the various purified thymic factors and a constructed peptide thereof on p15E-like factor-induced inhibition of monocyte polarization.

Figure 2 shows the results. The figure firstly depicts the inhibitory effect of a pool of LMWF <25kD, obtained from the serum of five patients with squamous cell carcinoma of the head and neck, on the fMLP-induced polarization of healthy donor monocytes. As can be seen, after addition of the chemoattractant fMLP, 28% of the healthy donor monocytes were polarized (mean, s.d. 3, n=8; figure 2, darkly hatched area); without addition of the chemoattractant a spontaneous polarization of only 3% of the monocytes was found (mean, s.d. 1, n=8). Addition of the pool of LMWF to this system resulted in a fMLP-induced polarization of only 14% (mean, s.d. 3, n=8; figure 2, lightly hatched area), which means an inhibition of 56%. This inhibition is due to p15E-like factors in the pool of serum LMWF, since after adsorption of the pool of LMWF with α-p15E mAb the pool no longer had inhibitory effects on the fMLP-induced polarization (mean 28% of monocyte polarization, n=2; data not shown). Figure 2 further shows the effects of different concentrations of purified and partially purified thymic hormone preparations (for characteristics, see Introduction, table 3) on the p15E-like factor-induced inhibition of monocyte polarization. The partially purified thymic hormone preparations TP-1 and TF5 were tested, as well as the purified hormones thymosin-α1, FTS-Zn, FTS-Zn plus an extra addition of 10% Zn, thymopoietin and the active site of thymopoietin, TP5. The peptide neurotensin and the solvent for thymopoietin (raffinose/glycine) were tested as controls. As can be seen, neurotensin and the solvent raffinose/glycine had no effect in restoring the p15E-like factor induced inhibition of monocyte polarization, while the neutralizing effect was optimal and complete when TP-1 was used at a concentration of 67 μg/ml culture fluid. The other hormones were less effective and results were variable. However, the optimally effective concentration of the purified hormones thymosin-α1 (6.7 μg/ml), FTS-Zn (6.7 μg/ml) and thymopoietin (2.2 μg/ml), and the pentapeptide TP5 (6.7 μg/ml) were lower in comparison to the optimal concentration of TP-1 (67 μg/ml). Although it has been described that thymulin (FTS-Zn) needs the addition of 10% Zn for the optimal effect of *in vitro* assays, the addition had a negative effect in our assay. It should be noted that neither TP-1, nor the other thymic hormone preparations had any effect on the spontaneous or fMLP-induced polarization of healthy donor monocytes as such, and that adsorption of the LMWF with an isotype control antibody had no effect on the inhibition.

With regard to the suggested more potent effect of TP-1 in comparison to the purified thymic hormones, we confirmed in another set of experiments, that the abrogating capacity of TP-1 was stronger than the pentapeptide TP5, using the LMWF

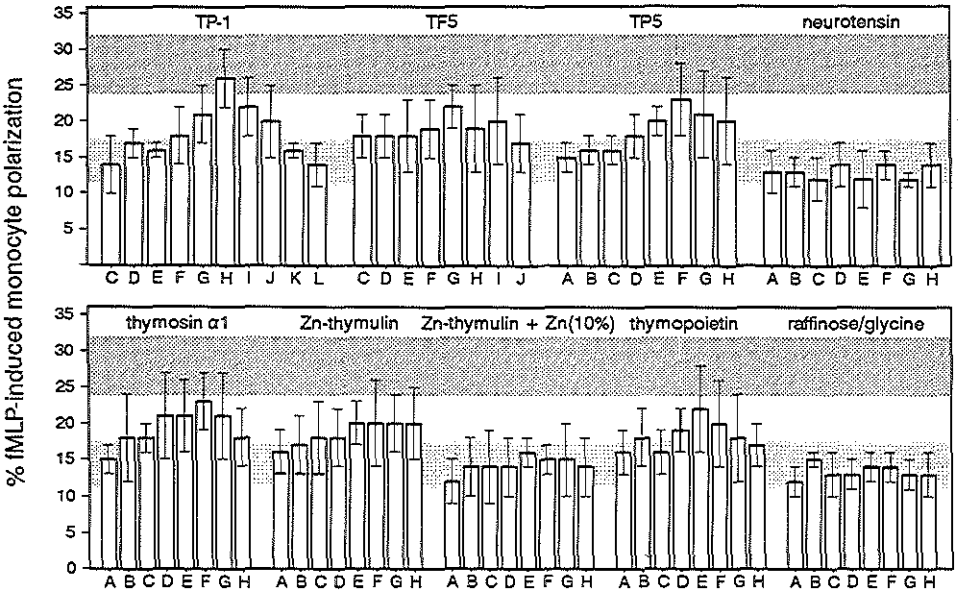

Figure 2. The effects of different concentrations of purified and partially purified thymic hormone preparations (for characteristics, see introduction) on the p15E-like factor induced inhibition of monocyte polarization. A = 22 ng/ml; B = 67 ng/ml; C = 0.22 μg/ml; D = 0.67 μg/ml; E = 2.2 μg/ml; F = 6.7 μg/ml; G = 22 μg/ml; H = 67 μg/ml; I = 0.22 mg/ml; J = 0.67 mg/ml; K = 2.2 mg/ml; L = 6.7 mg/ml.

of 17 patients with squamous cell carcinoma of the head and neck (paired Student's t-test; $p<0.001$; data not shown). It is not clear why the partially purified thymic hormone preparation TP-1 is more effective in neutralizing the immunosuppressive p15E-like activity. Kouttab et al. (98) showed that both TF5 and thymosin-α1 (one of the thymic hormones in TF5) were capable of enhancing the production of a B cell growth factor (BCGF-12kD) and T cell growth factor (TCGF; IL-2). Enhancement by thymosin-α1 could be obtained at 100-200-fold lower concentrations than seen with TF5, but the effects of TF5 were stronger in the optimal concentrations. The authors did not discuss this difference. In another study on TP-1 and two porcine thymic hormone preparations, the induction of lymphoproliferative responses and the enhancement of IL-2, IFN-γ and tumor necrosis factor (TNF) production of cord blood lymphocytes were compared (99). TP-1 and the porcine thymic hormone preparations were equally effective in the stimulation of lymphoproliferative responsiveness. However, TP-1 appeared to induce the highest amounts of IL-2, IFN-γ and TNF. The difference in immunostimulating effects and potencies that these thymic extracts show may not only reflect species differences but also differences in preparation procedures. Moreover, regarding the data presented in figure 2, it is possible that partially purified preparations may contain contaminants, or that different components in the thymic

extracts may act synergistically in the various biological activities of the thymic hormone preparations.

8.5 Mechanism of action of the thymic hormones in abrogating the p15E-like factor-induced immunosuppression

The mechanisms by which thymic hormone preparations abrogate p15E-like suppressive activity are not known and can only be speculated on. It has been reported that TF5 triggers the influx of Ca^{2+} and increases cGMP levels in thymocytes (100). The upregulation of signal transduction pathways by thymic factors could explain the counterregulation of p15E-like factors, for which a downregulation of signal transduction pathways has been described (see above). The upregulation of signal transduction pathways could also explain the influence on general cytokine production after stimulation with thymic hormones (see Introduction, table 4). Many consequences of a higher concentration of a local or peripheral cytokine production are imaginable.

The defective clustering of dendritic cells with allogeneic lymphocytes as found in head and neck squamous cell carcinoma patients and inducible with p15E-like factors was restored by the addition of thymic hormones *in vitro* (chapter 7). Scheeren et al. (101) have reported that this clustering of dendritic cells with lymphocytes is dependent on an LFA-1/ICAM-1 interaction. The intercellular adhesion molecule-1 (ICAM-1) is an adhesion molecule which is a ligand for the leukocyte function associated antigen-1 (LFA-1) (102,103), a member of the Integrin family (104) and therefore important in several LFA-1 dependent cellular interactions. These interactions are e.g. the adhesion of T cells to fibroblasts and endothelial cells (105) and the adhesion of human B cells to follicular dendritic cells (106). Since ICAM-1, which is normally expressed on vascular endothelial cells, thymic epithelial cells and fibroblasts (104,105), is expressed on many cell types in response to activation with cytokines like IFN-γ and TNF (104,107-109), one may speculate that the addition of TP-1 to dendritic cells with a defective clustering capability results in an upregulation of signal transduction, resulting in lymphokine production, expression of ICAM-1 and a consequent normal clustering capability.

Experiments on the mechanism by which TP-1 restores p15E-like factor-induced defective monocyte polarization have not yet been performed. In preliminary experiments we showed a marked increase of intracellular Ca^{2+} upon fMLP stimulation of monocytes, indicating the necessity of an upregulation of signal transduction for monocyte polarization. This influx of intracellular Ca^{2+} was decreased after preincubation with p15E-like factor containing LMWF, reflecting a downregulation of signal transduction. However, these preliminary experiments have not yet been reproduced and the effects of TP-1 in this system have not yet been tested. The question thus remains whether p15E is capable of downregulating the influx of intracellular Ca^{2+} in monocytes and which receptors can be responsible for this putative action. It is of interest that Pfeffer and Landsberger (110) recently reported that

IFN-α (sharing epitopes and activities with p15E, see above) decreased the fluidity of the plasma membrane lipid bilayer of human lymphoblastoid cells sensitive to the antiproliferative action of IFN-α. Although IFN-sensitive and IFN-resistant Daudi cell lines have similar numbers of high-affinity IFN-α receptors (111), IFN-resistant cells showed no decrease in membrane fluidity. IFN-α increased the rigidity of the plasma membrane lipid layer as early as 20 min after IFN-α addition, and plasma membrane rigidity returned to that of control cells within 5 h (110). The authors explained this early effect of IFN-α on membrane rigidity as a possible reflection of the cooperative interaction of cell-surface IFN-α receptors induced by IFN-α binding to these receptors. Another suggestion was that the membrane effects were caused via intracellular signals generated by IFN-α-receptor stimulation. Since the action of p15E and IFN-α on monocyte polarization (see above) are similar and early processes, the actions of p15E could involve the same receptors and processes as described for IFN-α. It is also possible that p15E, being a transmembrane envelope protein with hydrophobic characteristics, influences the rigidity of the membrane via a simple insertion.

The question remains via which mechanism thymic hormones are capable of restoring the p15E-induced defective polarization of monocytes. Recently, Safer et al. (112) reported that polymerization of actin in highly motile cells such as blood platelets and neutrophils is dependent on thymosin-β4. Thymosin-β4 is a thymic factor, but is not exclusive to thymic tissue, occurring in numerous tissues (113). Thymosin-β4 inhibits the salt-induced polymerization of actin by sequestering monomers, and can be covalently coupled to G-actin by the zero-length cross-linker 1-ethyl-3-(3-dimethyl)-aminopropyl carbodiimide (114) and thereby promotes cell locomotion by influencing the actin dynamics in highly motile cells (115). It remains to be clarified whether other thymic peptides share this function with thymosin-β4 or whether this function is related to the restoration of defective monocyte polarization.

In conclusion, thymic hormones not only have immunomodulating effects by restoring defective T cell functions in patients with defects in CMI, but also by restoring defective functions of cells of the monocyte/dendritic cell system. These effects are mainly achieved by abrogating the activities of cytokine-like endogenously produced factors structurally related to retroviral p15E and affecting monocyte polarization and the clustering capability of dendritic cells. The mechanisms of action of p15E and the thymic hormones need further investigation.

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SUMMARY

This thesis describes the immunosuppression due to factors structurally related to retroviral proteins and the possibities to interfere with this suppression by thymic hormones.

Immunosuppression

Defective functioning of the immune system can be the consequence of external influences such as a viral infection. This may cause an increased susceptibility for infections, an increased incidence of malignancies and of autoimmune disease.

In recent years much attention has been paid to the human immunodeficiency virus (HIV), the etiologic agent of the acquired immunodeficiency syndrome (AIDS). This virus has the capability of eventually incapacitating the immune system, resulting in opportunistic infections and often malignant disease.

Some knowledge of the functioning of the immune system is required to understand the different mechanisms involved in immunosuppression.

Immunoreactivity can be divided into the cellular and the humoral immunity. The humoral immunity is characterized by soluble serum factors and the production of antibodies against foreign antigens. A historical example of humoral immunity is the resistance to the human pox virus. After vaccination with cow pox the so called B lymphocytes produce antibodies recognizing and eliminating the human pox virus.

The cellular immunity is mediated by T lymphocytes, monocytes, macrophages and dendritic cells (DC). DC play an important role by processing and presenting antigens to lymphocytes. Subsequently, the lymphocytes will be able to recognize an antigen. After recognition T-helper lymphocytes become activated and produce lymphokines to activate other immune cells: B cells, for instance, will proliferate and transform to produce antibodies which recognize the antigen. This indicates that humoral immunity and cellular immunity are interrelated. Monocytes play an important role by migrating to the site of infection where these cells mature into macrophages. Macrophages exhibit several functions, amongst which the phagocytosis and killing of bacteria, the killing of cancer cells, and the activation of other immune cells.

The interaction between cells and the production of lymphokines are major processes in the physiology of the immune system. Monocytes are important cytokine producing cells, while intercellular contact and communication are typical DC functions. This thesis describes a defective function of monocytes and DC in different diseases characterized by defects in cell-mediated immunity (CMI), namely AIDS, chronic infection of the upper airways (chronic purulent rhinosinusitis), squamous carcinoma of the head and neck (cancer in the oral cavity, larynx or oropharynx), as well as some autoimmune diseases (Graves' disease, insulin-

dependent diabetes mellitus (IDDM) and Sjögren's syndrome (SS)).

Immunosuppression by retrovirus-related factors

Several mechanisms are involved in the immunosuppression caused by HIV infection:

1) HIV infects T lymphocytes expressing the cell-surface CD4 molecule. After binding CD4, HIV can be internalized and finally integrated in the cellular genome, resulting in functional defects and destruction of the lymphocytes.
2) Monocytes and dendritic cells expressing low amounts of CD4 are infected as well. These cells are thought to function as a reservoir and vehicle for infection, and show several functional defects in HIV infection. This clearly may have consequences for the functioning of the immune system.
3) Certain retroviral proteins possess immunosuppressive properties. Especially the transmembrane (TM-)proteins have been reported to suppress the function of lymphocytes, monocytes and dendritic cells.

The immunosuppressive properties of retroviral TM-proteins have been extensively described in animal models. Infection with feline and murine leukemia viruses may cause leukemia in cats and mice, respectively. The infection can be accompanied by immunosuppression characterized by defects in CMI, most notably a decreased monocyte chemotaxis. It has become clear that the TM-protein p15E is, at least partly, responsible for the immunodeficiency. In humans infected with HIV the TM-protein gp41 exerts similar immunosuppressive effects as murine or feline p15E.

This thesis describes the immunosuppressive effects of TM-proteins and related factors. The main assays used are the monocyte polarization and the clustering capability of dendritic cells. The polarization assay is a rapid and easy method to measure monocyte chemotactic responsiveness and is based on the changes in the shape of monocytes ('polarization') under the influence of a chemoattractant. This process can be related to the capability of monocytes to migrate to the site of infection. The clustering capability of dendritic cells is the capability of these cells to form homocytotropic clusters and clusters with lymphocytes, thereby creating a microenvironment suitable for antigen presentation and activation of T- and B-lymphocytes.

Chapter 1

Chapter 1 is an introduction on immunosuppressive effects due to retrovirus-related factors. The consequences of retroviral infections are described as well as the immunosuppressive properties of TM-proteins and a synthetic peptide homologous to a conserved sequence in p15E. The detection of p15E-like factors in human tissue and the possible consequences of endogenous retroviruses in the genome of mice and humans are discussed.

Chapter 1 is also an introduction on the properties of thymic hormone

preparations and thymic hormones and their possible influence on immunosuppressive effects due to p15E-like factors.

Chapter 2

Chapter 2 describes a monocyte polarization inhibiting factor in serum of HIV-infected men. This factor, detectable in asymptomatic HIV-infected individuals, AIDS-related complex (ARC) and AIDS patients, shares epitopes with the HIV TM-protein gp41, since monoclonal antibodies (mAb) to HIV gp41 were able to adsorb the immunosuppressive factor from the serum. In other adsorption experiments, mAb to p15E had the same effects as anti-gp41, which indicates that gp41 may share epitopes with p15E.

Chapter 3

In chapter 3 the presence of an immunosuppressive p15E-like factor in the serum of patients with unexplained relapsing chronic purulent infections of the upper airways is described. The presence of the factor in the serum was associated with the existence of defects in CMI (disturbances in delayed-type hypersensitivity reaction, the production of macrophage migration inhibition factor and monocyte polarization).

Chapter 4

Chapter 4 describes the defects in monocyte polarization and dendritic cell clustering in patients with Graves' disease. These defects could be ascribed to the presence of immunosuppressive p15E-like factors in the serum of these patients. A physiological role of non-specific endogenous p15E-like factors is discussed.

Chapter 5

In chapter 5 a prospective follow-up study is described on the levels of bioactive p15E-like serum factors during a period of two years in head and neck squamous cell carcinoma (HNSCC) patients with or without recurrent/residual disease after therapy with curative intent. The study strongly supports the concept that HNSCC tumors are the production site of p15E-like immunosuppressive factors and indicates that the positivity for p15E-like factors may be used for future studies on early serum markers for recurrent disease developing in the first year after treatment.

Chapter 6

Chapter 6 describes the *in vivo* effects of the thymic hormone preparation TP-1 in a double-blind cross-over trial in patients suffering from chronic purulent rhinosinusitis resistant to the current therapies of surgery and treatment with antibiotics. Defects in monocyte polarization were restored and bioactive p15E-like factors in serum were neutralized by TP-1. Beneficial effects on the clinical condition, the purulent nasal discharge and the bacterial invasion are reported.

Chapter 7

In chapter 7 the *in vitro* effects of TP-1 on monocytes and DC isolated from the peripheral blood of HNSCC patients are described. Defective polarization of monocytes and the defective clustering capability of dendritic cells could be restored by TP-1. The *in vitro* influence of TP-1 on the suppression exerted by p15E-like serum factors on monocyte and dendritic cell function was established as well.

Chapter 8

In chapter 8 the properties of p15E-like serum factors (which are responsible for disturbances in CMI) and the effects of thymic hormone preparations and thymic hormones are discussed. Reports on amino acid sequences of p15E and gp41 in relation to their regulatory activity on the immune system are reviewed. The putative origin of p15E-like factors in the serum of patients with HNSCC, Graves' disease, Sjögren's syndrome, insulin-dependent diabetes mellitus and chronic purulent rhinosinusitis is discussed, as well as the possible role of p15E-like factors in the regulation of immune homeostasis.

Particular defects in the functions of T lymphocytes, monocytes and dendritic cells can be restored by thymic hormones. These effects are mainly achieved by abrogating the activities of cytokine-like endogenously produced factors structurally related to retroviral p15E, which affect monocyte polarization and the clustering capability of dendritic cells. The mechanisms of action of p15E and the thymic hormones need further investigation.

SAMENVATTING

Dit proefschrift behandelt de verlaging van de weerstand (immunosuppressie) door factoren die lijken op eiwitten van retrovirussen en de mogelijke therapie met hormonen afkomstig van de zwezerik (thymus).

Immunosuppressie

Het uit balans raken van het immuunsysteem kan het gevolg zijn van invloeden van buitenaf, zoals een virusinfectie, en kan zich uiten als een verhoogde infectiegevoeligheid, en een verhoogde kans op kanker of autoimmuunziekten (bij autoimmuunziekte valt het immuunsysteem het eigen lichaam aan).

Eén van de bekendste voorbeelden van immunosuppressie is het "acquired immunodeficiency syndrome" (AIDS), veroorzaakt door het "human immunodeficiency virus" (HIV). Infectie met HIV veroorzaakt een verstoring van het immuunsysteem, waardoor opportunistische infecties kunnen optreden. Het betreft hier infecties met bacteriën waar we normaal gesproken niet ziek van worden. Zo kan bijvoorbeeld een ernstige longontsteking ontstaan. Een ander mogelijk gevolg van de immunosuppressie is een verhoogde kans op kanker. De paar kankercellen die in een gezond lichaam door het immuunsysteem aangepakt zouden worden, krijgen nu de kans om door te groeien en ander weefsel te overwoekeren. Om de processen van immunosuppressie te kunnen volgen is enige kennis van het immuunsysteem noodzakelijk.

Immuniteit kan worden onderverdeeld in de cellulaire en de humorale immuniteit. Onder de humorale immuniteit wordt verstaan de weerstand tegen lichaamsvreemd materiaal (virussen, bacteriën, parasieten, chemische stoffen etc.) door antistoffen en andere factoren. Een klassiek voorbeeld van humorale immuniteit is de afweer tegen pokken, die verkregen is door vaccinatie met koepokken. Na inenting met koepokken (die niet schadelijk zijn voor mensen), worden antistoffen aangemaakt door bepaalde witte bloedlichaampjes (de zogenaamde B-lymfocyten). Bij infectie met pokken herkennen de antistoffen tegen de koepokken ook het menselijke pokkenvirus, zodat dat snel opgeruimd kan worden.

Onder cellulaire immuniteit wordt verstaan de weerstand tegen lichaamsvreemd materiaal door de andere witte bloedlichaampjes van het immuunsysteem (T-lymfocyten, granulocyten, monocyten, macrofagen en dendritische cellen). Een belangrijke eigenschap van monocyten is dat ze vanuit het bloed naar de plaats van ontsteking kunnen migreren om daar uit te rijpen tot macrofagen. Macrofagen hebben verschillende functies zoals het opeten van bacteriën, het doden van kankercellen en het activeren van andere cellen van het immuunsysteem. Dendritische cellen hebben als voornaamste functie het verwerken van lichaamsvreemd materiaal om het vervolgens aan te kunnen bieden aan T-helper lymfocyten. Deze lymfocyten herkennen dan het lichaamsvreemde

materiaal (dit kan ook een kankercel of een virus-geïnfecteerde cel zijn, omdat die andere kenmerken hebben dan normale cellen in het lichaam). Als gevolg van herkenning van de lichaamsvreemde stof (antigeen) worden T-helper lymfocyten geactiveerd om hulp te bieden aan andere immuun-cellen ter eliminatie van het antigeen: T-cytotoxische lymfocyten kunnen na herkenning van het antigeen virus-geïnfecteerde cellen of kankercellen doden; de B-cellen kunnen met de hulp van de T-helper lymfocyten antistoffen maken tegen het antigeen. Hieruit blijkt dat B-cellen afhankelijk kunnen zijn van de T-cellen. Dit is slechts van één van de voorbeelden van het verfijnde samenspel tussen de verschillende cellen van het immuunsysteem.

Monocyten en dendritische cellen vervullen een belangrijke rol aan de basis van al deze immuunprocessen. In dit proefschrift wordt met name aandacht besteed aan functiestoornissen van deze cellen bij verschillende ziektebeelden waarbij immunosuppressie een rol speelt, namelijk AIDS, chronische infecties van de bovenste luchtwegen (chronische purulente rhinosinusitis), autoimmuunziekten (de ziekte van Graves, diabetes mellitus type 1 (een bepaalde vorm van suikerziekte) en Sjögren syndroom) en kanker in het hoofd-halsgebied.

Immunosuppressie door retroviraal gerelateerde factoren

De immunosuppressie, zoals aangetroffen bij AIDS, kan op een aantal manieren verklaard worden:

(1) Het is bekend dat HIV met name de T-helper lymfocyten infecteert, doordat deze cellen een bepaald eiwit (CD4) op hun membraan tot expressie brengen waarop het HIV-virus kan aanhechten. Deze geïnfecteerde T-helper lymfocyten kunnen daardoor hun belangrijke functies in het immuunsysteem niet meer naar behoren uitoefenen. In de cellen kan het virus zich vermenigvuldigen en ze zullen afsterven als de nieuwe virussen vrijkomen.

(2) Andere cellen van het immuunsysteem die ook CD4 tot expressie brengen, zoals monocyten, macrofagen en dendritische cellen, kunnen besmet raken met HIV. Infectie van deze cellen kan belangrijke gevolgen hebben voor het functioneren van het immuunsysteem.

(3) Het is gebleken dat bepaalde eiwitten van retrovirussen immunosuppressieve capaciteiten bezitten. Met name de eiwitten die door de membraan van het virus steken (de transmembraan (TM)-eiwitten) kunnen functiestoornissen van allerlei cellen van het immuunsysteem bewerkstelligen.

Uitgebreid onderzoek naar immunosuppressieve TM-eiwitten van retrovirussen is verricht bij muizen en katten. Bij katten en muizen die geïnfecteerd raken met bepaalde retrovirussen (virussen die op HIV lijken) en hierdoor bloedkanker (leukemie) kunnen krijgen, is er tevens sprake van een vorm van immuunsuppressie. Deze wordt onder andere gekarakteriseerd door een verslechterde functie van monocyten, die met name veroorzaakt wordt door het TM-eiwit p15E. Bij HIV-infectie bij mensen is gebleken dat het TM-eiwit gp41 dezelfde soort effecten kan hebben.

In dit proefschrift wordt een aantal immunosuppressieve effecten van TM-eiwitten en factoren die daarop lijken, beschreven. Als testsystemen zijn de polarisatie van monocyten en het clusterend vermogen van dendritische cellen gebruikt. Polarisatie van monocyten is het veranderen van hun vorm onder invloed van een bepaalde chemische stof. Dit proces kan gerelateerd worden aan het vermogen van monocyten om naar een ontsteking te migreren. Het clusterend vermogen van dendritische cellen is het vermogen van deze cellen om met elkaar en met andere cellen clusters (groepjes) te vormen, zodat een micromilieu ontstaat waarin optimale communicatie tussen de cellen mogelijk is en de cellen geactiveerd kunnen worden voor hun functies in het immuunsysteem.

Hoofdstuk 1

In hoofdstuk 1 van dit proefschrift wordt een inleiding gegeven op het verrichte onderzoek naar immunosuppressie door retrovirus-gerelateerde factoren. De gevolgen van infecties met retrovirussen worden belicht en er wordt besproken welke stukken van de TM-eiwitten van belang kunnen zijn voor immunosuppressie. Tevens wordt aandacht besteed aan het voorkomen en de eventuele gevolgen van de zogenaamde endogene retrovirussen en van p15E-achtige factoren. Endogene retro-virussen zijn retrovirussen die in onze genetische informatie (DNA) zijn opgenomen door infectie van de ei- of zaadcellen van onze voorouders. Wij blijken retroviraal materiaal in ons DNA te bezitten zonder dat van infectie met zogenaamde exogene retrovirussen, zoals HIV, sprake is. Mogelijk kan immunosuppressie ontstaan nadat stukken DNA met endogeen retroviraal materiaal, of DNA dat hier sterk op lijkt, in werking zijn gesteld (expressie), waardoor eiwitten die lijken op p15E of gp41 geproduceerd worden. Deze eiwitten kan men dan p15E-achtige factoren noemen. Het is bijvoorbeeld beschreven dat p15E-achtige factoren door kanker-cellen gemaakt kunnen worden, zonder dat aangetoond kon worden dat deze cellen geïnfecteerd waren met een retrovirus.

In hoofdstuk 1 wordt tevens een inleiding gegeven op de werkzaamheid van de verschillende thymushormoonpreparaten en thymushormonen in verband met het mogelijke effect op door p15E-achtige factoren veroorzaakte immuno-suppressie.

Hoofdstuk 2

In hoofdstuk 2 van dit proefschrift wordt beschreven dat in het bloed van HIV-geïnfecteerde mensen factoren circuleren die gerelateerd kunnen worden aan het TM-eiwit gp41 van HIV. Fracties van het bloed zijn getest op remming van het polariserend vermogen van monocyten, afkomstig van gezonde donoren. Het bleek dat alleen de fracties van HIV-geïnfecteerde mensen, maar niet die van gezonde controles, de monocytpolarisatie remden. Met antistoffen, die specifiek gericht zijn tegen gp41, kon de factor die de remming veroorzaakte, uit de fracties van het bloed weggehaald worden. Geïnactiveerd virus (een virus dat zo behandeld is dat het geen cellen meer kan infecteren, maar met behoud van belangrijke, herkenbare

structuren) en artificieel gemaakt gp41 waren eveneens in staat remming van de monocytchemotaxis te bewerkstelligen. Niet alleen met specifieke antistoffen tegen gp41 van HIV, maar ook met specifieke antistoffen gericht tegen het TM-eiwit p15E van muize-retrovirus, kon deze remming opgeheven worden.

Geconcludeerd wordt dat in het bloed van HIV-positieve personen gp41-achtige factoren circuleren, die de monocytpolarisatie remmen (dus immuunsuppressieve capaciteiten bezitten) en tevens overeenkomsten vertonen met p15E van muize-retrovirus.

Hoofdstuk 3

In hoofdstuk 3 wordt beschreven dat de functiestoornissen van lymfocyten en monocyten bij patiënten met chronische ontstekingen in de bovenste luchtwegen gerelateerd kunnen worden aan de aanwezigheid van p15E-achtige eiwitten in het bloed. De oorsprong van deze circulerende factoren is onbekend. Hierbij kan gedacht worden aan een infectie met een nog onbekend retrovirus of een verhoogde produktie van endogeen retroviraal-gerelateerde eiwitten ter regulering van de balans van het immuunsysteem.

Hoofdstuk 4

In hoofdstuk 4 worden stoornissen in de polarisatie van monocyten en het clusterend vermogen van dendritische cellen van patiënten met de ziekte van Graves beschreven. De ziekte van Graves is een autoimmuunziekte die wordt gekarakteriseerd door overstimulatie van de schildklier door de aanwezigheid van schildklierstimulerende antistoffen in het bloed. Exophthalmus (uitpuilende ogen) en/of struma (vergrote schildklier), nervositeit en warmte-intolerantie behoren tot de symptomen.

De bovengenoemde cellulaire immuunstoornissen die we bij deze patiënten hebben gevonden kunnen worden gerelateerd aan de aanwezigheid van p15E-achtige factoren in het bloed van deze patiënten.

Hoofdstuk 5

Hoofdstuk 5 behandelt een studie naar de bioactiviteit van p15E-achtige serum-factoren bij patiënten met een tumor (plaveiselcelcarcinoom) in het hoofd/halsgebied (HNSCC). Deze patiënten zijn vervolgd in de tijd van voor de behandeling tot twee jaar na behandeling van de tumor.

Deze studie ondersteunt het concept dat HNSCC een bron van p15E-achtige factoren is en geeft aan dat de detectie van deze serumfactoren gebruikt kan worden voor meer uitgebreide studies naar mogelijkheden om te voorspellen of na een therapeutische behandeling de tumor weer is gaan groeien.

Hoofdstuk 6

In hoofdstuk 6 worden de effecten van een behandeling met het thymus-hormoonpreparaat TP-1 in patiënten met chronische purulente rhinosinusitis

beschreven. Bij deze patiënten zijn conservatieve (antibiotica) en chirurgische therapie zonder succes gebleven.

Tijdens de behandeling met TP-1 herstelde de monocytpolarisatie zich en verdwenen de p15E-achtige factoren uit het bloed. Er trad tevens een sterke klinische verbetering op; de nasale afscheiding verminderde en bacteriekweken werden negatief.

Hoofdstuk 7

In hoofdstuk 7 worden de *in vitro* effecten van TP-1 op monocyten en dendritische cellen van HNSCC patiënten beschreven. De stoornissen in monocyt-polarisatie en clustering van dendritische cellen konden worden opgeheven door TP-1. Tevens kon worden aangetoond dat de door p15E-achtige factoren verkregen remming op monocytpolarisatie en dendrietclustering door *in vitro* toediening van TP-1 kon worden teniet gedaan.

Hoofdstuk 8

In dit hoofdstuk wordt de herkomst en functie van p15E-achtige factoren besproken en wordt de invloed van thymushormonen hierop bediscussieerd.

Er wordt een overzicht gegeven van aminozuursequenties van p15E en gp41 in relatie tot hun immuunregulerende activiteiten. De vermoedelijke herkomst van p15E-achtige factoren in HNSCC, chronische purulente rhinosinusitis, de ziekte van Graves, diabetes mellitus type 1 en Sjögren syndroom, en de mogelijke immuun-regulatoire functies van deze factoren worden besproken.

Zowel *in vivo* als *in vitro* kunnen thymushormonen bepaalde functiestoornissen van T lymfocyten, monocyten en dendritische cellen herstellen. Deze effecten lijken vooral te worden veroorzaakt doordat deze thymushormonen de bioactiviteit van de p15E-achtige factoren onderdrukken.

ABBREVIATIONS

aa	amino acids
AIDS	acquired immunodeficiency syndrome
AHS	zymosan-activated human serum
AKR-MuLV	AKR murine leukemia virus
ALAT	alanine acid transferrin
ANA	antinuclear antibodies
ARC	AIDS-related complex
ASAT	aspartic acid transferrin
ATL	adult T cell leukemia
BCGF	B cell growth factor
BLV	bovine leukemia virus
BSA	bovine serum albumin
BSS	balanced salt solution
CA	major capsid protein
cGMP	cyclic guanine monophosphate
CMI	cell-mediated immunity
ConA	concanavalin A
CPR	chronic purulent rhinosinusitis
CSF	colony stimulating factor
DC	dendritic cell(s)
DTH	delayed type hypersensitivity
ESR	erythrocyte sedimentation rate
FeLV	feline leukemia virus
FeSV	feline sarcoma virus
fMLP	N-formyl-methionyl-leucyl-phenylalanine
F-MuLV	Friend murine leukemia virus
FTS-Zn	thymulin
HFV	human foamy virus
HIV-1 (-2)	human immunodeficiency virus type 1 (type 2)
HLA	human leukocyte antigen
HNSCC	head and neck squamous cell carcinoma
HTLV-1 (-2)	human T-lymphotropic virus type 1 (type 2)
ICAM-1	intercellular adhesion molecule-1
IDDM	insulin-dependent diabetes mellitus
IFN	interferon
IFN-α	interferon alpha
IFN-γ	interferon gamma
IL-1-β	interleukin 1 beta
IL-2	interleukin 2
IL-2-R	interleukin 2 receptor
IL-3	interleukin 3
JRA	juvenile rheumatoid arthritis
kD	kilodalton
LDCF	lymphocyte-derived chemotactic factor
LFA-1	leukocyte-function associated antigen-1
LMWF	low molecular weight factor(s)

M-MuLV	Moloney murine leukemia virus
mAb	monoclonal antibody(ies)
MCF	Mink cell focus-forming
MHC	major histocompatibility complex
MIF	migration inhibition factor
MLR	mixed leukocyte reaction
MNL	mononuclear leukocyte
MOPS	3-(N-morpholino) propanesulphanic acid
MPMV	Mason-Pfizer monkey virus
MuLV	murine leukemia virus(es)
NK cell	natural killer cell
NSE	non-specific esterase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHA	phytohemagglutinin
PKC	protein kinase C
PWM	pokeweed mitogen
RAST	radioallergosorbent test
R-MuLV	murine Rauscher leukemia virus
RLA	rat liver araginase
rDNA gp41	recombinant DNA gp41
rp15E	recombinant p15E
RSV	Rous sarcoma virus
SFV	simian foamy virus
SKSD	streptokinase-streptodornase
SLE	systemic lupus erythematosus
SMRV-H	squirrel monkey-like virus, of human lymphoblastoid cell line
SS	Sjögren's syndrome
SS-A	Sjögren's syndrome Ro-antigen
SS-B	Sjögren's syndrome La-antigen
TBII	thyrotropin binding inhibiting immunoglobulin
TCGF	T cell growth factor
TcR	T cell receptor
TF5	thymosin fraction V
TFX	thymic factor X
THF	thymic humoral factor
TNF	tumor necrosis factor
TM-protein(s)	transmembrane envelope protein(s)
TP-1	thymostimulin
TP5	thymopentin
TRH	thyrotropin-releasing hormone
TSH	thyroid stimulating hormone
TSH-R	TSH receptor

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CURRICULUM VITAE

Maarten Pieter Rinse Tas

03-03-1961 : Geboren te 's-Gravenhage

1973-1979 : Gymnasium B

1979-1984 : Candidaats Medische Biologie

1984-1987 : Doctoraal Medische Biologie
Hoofdvak: - Immunologie, afd. Pathologie, RIVM, Bilthoven
Bijvakken: - Klinische Immunologie, afd. Pathologie, Vrije Universiteit Amsterdam
- Virologie, afd. Virologie, Universiteit van Amsterdam
Extra cursussen: - Informatica
- Didactiek
- Radioactiviteit

1987 : Project-assistent Informatiekunde, Universiteit Nijenrode Breukelen
Project: - Informatievoorziening bij grote bedrijven

1987-1989 : Assistent-onderzoeker, Vrije Universiteit Amsterdam
Projecten: - De effecten van thymushormonen op de cellulaire immuniteit in patiënten met chronische infecties van de bovenste luchtwegen; een "double-blind cross-over trial"
- De effecten van thymushormonen op de cellulaire immuniteit in patiënten met een plaveiselcelcarcinoom in het hoofd-halsgebied; een "dose-finding study"*
Cursus: - Immunologie voor gevorderden
Onderwijs: - Immunologie voor gevorderden

1989-heden : Wetenschappelijk onderzoeker, Erasmus Universiteit Rotterdam
Projecten: - De effecten van thymushormonen op de cellulaire immuniteit in patiënten met een plaveiselcelcarcinoom in het hoofd-halsgebied; een "dose-finding study" (vervolg *)
- De effecten van thymushormonen op de cellulaire immuniteit in patiënten met een plaveiselcelcarcinoom in het hoofd-halsgebied; opzet van een "double-blind pilot study"
Onderwijs: - Immunologie voor gevorderden
- Praktikumassistent Immunologie & Histologie

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