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Immunoregulation by Thymopoietin

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The immune system is a major component of the host defenses and is involved in responses to diverse extrinsic influences. Lymphocytes in all of their diversity create the finely tuned reactions characteristic of specific immunity. Following the recognition that lymphocytes could be classified into thymus-dependent T cells and thymus-independent B cells, contemporary immunology has progressed further and further in classifying subsets of T cells and B cells; the immune system is now revealed to be a cellular hierarchy of effector cells controlled in turn by arrays of regulatory cells.

CELLULAR CIRCUITRY IN THE IMMUNE SYSTEM

One important step in the dissection of cellular circuitry in the immune system has been the recognition that discrete differentiative and functional stages of lymphocyte differentiation are associated with distinct cell surface molecules. This was first established in the mouse, wherein allo-antisera were generated that first distinguished T cells and B cells and subsequently distinguished subclasses of T cells [1]. With these reagents, it was established that discrete cells, with stable cell surface phenotypes, were involved in both T effector functions and regulation. Thus the final response of the immune system seems more dependent on the balance of inducer and inhibitory cells than upon the actual level of effector cells. These analyses have progressed, and it is now clear that the immune response involves a dynamic and continually evolving dialogue between a number of cells. Thus immunoregulation can be considered at a cellular level, with analyses of the different cell types involved during the evolution of an immune response, and it can also be considered at a chemical level in terms of the molecules involved in these regulations. Chemical signals in turn can involve molecules used for communication and interaction between cells – for example, helper or suppressor factors – or hormonal signals regulating the overall balance of immune responsiveness – for example, thymopoietin – as described below [2, 3].

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OKT MONOCLONAL ANTIBODIES TO HUMAN T CELLS

The general concept that cellular circuitry of the immune system is involved in maintaining immunoregulation was established in the mouse, and it is now possible to extend these analyses to the human immune system by using monoclonal antibodies generated by the hybridoma technique of Kohler and Milstein [4]. A series of monoclonal antibodies generated in our laboratory (the OKT series) have been characterized as identifying discrete differentiative and functional stages of T cells in man [5, 6]. In particular, circulating T cells are identified by a number of monoclonal antibodies, including OKT1, OKT3, and OKT11 [7–9]. In normal individuals approximately 60% of peripheral T cells are identified by a monoclonal antibody termed OKT4, and this subclass contains the inducer T cell population that produces helper factors that positively induce effector functions in both T cells and B cells [10]. A reciprocal population of circulating T cells is identified by OKT8 (and largely by OKT5), and this subclass contains suppressor cells for both T and B cell functions, plus cytotoxic T cells [11]. With further study employing irradiation, it can be shown that the $OKT4^+$ population contains a radiosensitive subclass that is required for the suppressive activities of $OKT8^+$ cells [12]. Thus it is clear that dissection with monoclonal antibodies is revealing that the human immune system has hierarchies of control, as described in the mouse. The monoclonal antibodies being used to detect such mechanisms are not only useful in such physiological studies but also have direct application to the analysis of the alterations in immunoregulation that occur in human disease and the effects of putative immunoregulatory compounds [11, 13, 14].

THE THYMUS

The thymus consists of an epithelial stroma, derived from the third branchial arch, into which immigrant prothymocytes derived from the bone marrow enter, differentiate, and finally leave as differentiated T cells [2, 15, 16]. The literature describing attempts to isolate putative thymic hormones secreted by the epithelial cells of the thymus to regulate T cell differentiation is extensive. Amino acid sequences for candidate thymic hormones have been published by three groups [17–19]. A.L. Goldstein et al have extensively studied thymic extracts and used the term thymosins for the multiple bands obtained when crude thymic extracts are fractionated by isoelectric focusing. Sequences have been published for an acidic protein termed α 1 thymosin [20, 21], and a more basic protein termed β 1 thymosin [22], although the amino acid sequence of the latter molecule is identical with the previously published sequence of ubiquitin, a nonhistone protein that is found in virtually all tissues [23]. This would suggest that it is inappropriate to designate such proteins as "thymosins" simply on the basis of their isolation from a thymus extract. Furthermore, scant evidence has been presented for the hormonal nature of α 1 thymosin.

Facteur thymic serique (FTS) is a nonapeptide that was isolated and sequenced, by J-F. Bach and colleagues [24], which has subsequently been synthesized [25]. It disappears from serum after thymectomy, as measured by bioassay, and has recently been localized in epithelial cells of the thymus [26]. Of interest is the finding that, although its chemistry is quite distinct from that of thymopoietin, it acts similarly, but not identically, in a number of bioassay systems [27].

Thymopoietin is a 49 amino acid polypeptide, and its chemistry and biology are described more fully below.

THYMOPOIETIN – ISOLATION

Thymopoietin was isolated by its effect on neuromuscular transmission [28, 29]. This effect was detected in studies related to the human disease myasthenia gravis, wherein neuromuscular impairment is associated with thymic abnormalities [20, 30]. Analysis of these alterations led to the hypothesis that autoimmune thymitis is present in myasthenia gravis. Experimental analysis of autoimmune thymitis induced in mice, rats, and guinea pigs suggested that the neuromuscular impairment was related to a substance released by the thymus [28, 32, 33]. Furthermore, analysis of neuromuscular transmission in thymectomized and thymus-grafted rats again suggested that a substance inhibiting neuromuscular transmission was being secreted normally by the thymus [34, 35]. Although no curare-like effect could be detected in thymus extracts, in conformity with earlier studies it was found that thymus extracts, when injected into animals, resulted in a slight but detectable inhibition of neuromuscular transmission after a period of 24 hours [17, 36]. This assay was therefore used to monitor the fractionation of thymus extracts for the isolation of thymopoietin. Two closely related peptides termed thymopoietin I and thymopoietin II were isolated, these purified molecules having only quantitative differences in their actions on neuromuscular transmission [16, 17].

THYMOPOIETIN – STRUCTURE

Thymopoietin is a 49 amino acid polypeptide chain whose complete amino acid sequence has been reported [37]. Thymopoietin I differs from thymopoietin II by one amino acid only [unpublished observation], and this change does not involve the pentapeptide active site. A tridecapeptide corresponding to residues 29 through 41 was synthesized and shown to be biologically active. Subsequently, a pentapeptide fragment (Arg-Lys-Asp-Val-Tyr) corresponding to residues 32–36 and termed TP 5, was synthesized. It displayed the biological activities of thymopoietin and thus probably represents an "active site" of thymopoietin [38–40]. TP-5 has been used in many of the biological studies described below and is now being evaluated as an immunoregulatory compound for therapeutic use in man [41].

THYMOPOIETIN – INDUCTIVE DIFFERENTIATIVE EFFECTS

The earliest studies of biological effects of thymopoietin in relation to the development of the immune system were focused on its inductive effects on early T cell lineage [42–44]. Prothymocytes in the mouse were defined as cells lacking the Thy-1 surface marker, which is characteristic of thymocytes and peripheral T cells, and as cells having the capability of repopulating the thymus in lethally irradiated mice. In populations enriched for prothymocytes from murine spleen or bone marrow, it was shown that thymopoietin selectively induced the differentiation of prothymocytes to thymocytes as measured by cell surface phenotypic changes and the development of mitogenic responses [16, 40]. Analysis of the mode of action of thymopoietin implicated a cyclic AMP second signal and the involvement of transcription of DNA to RNA and translation of RNA to protein, although it was not formally shown that the new cell surface antigens displayed were synthesized de novo [43, 45, 46]. Furthermore, it was established that thymopoietin triggered these processes, being required but briefly, and that following this induction, differentiation proceeded in the absence of any further inductive stimulus [40, 47]. These effects of thymopoietin could also be demonstrated in vivo in experiments on nude mice [48–50].

Since induction of prothymocyte to thymocyte differentiation appeared to involve a cyclic AMP signal, it was predicted that other agents that elevated intracellular cyclic AMP levels would also trigger differentiation, and this was indeed found to be the case [27]. Furthermore, B cell differentiation also appeared to involve cyclic AMP as an intracellular signal. This finding was used to distinguish selective from nonselective inducing agents by running parallel assays of B cell and T cell differentiation. Thymopoietin selectively induced T cell differentiation and actually inhibited B cell differentiation [40, 44]. This selective inductive capacity of thymopoietin for prothymocyte to thymocyte differentiation was used in defining some properties of prothymocytes. For example, it was shown that, following spleen or bone marrow grafts in lethally irradiated mice, the thymus was repopulated by donor cells that were initially Thy-1$^-$ but were induced in vitro by thymopoietin to become Thy-1$^+$ [42, 51]. Furthermore, a large proportion of terminal deoxynucleotide transferase containing cells in the bone marrow were also Thy-1$^-$ and could be induced to Thy-1$^+$ by thymopoietin [43].

These early T cell differentiative steps were studied extensively in mice because of the availability of allo-antisera and congeneic mouse controls, but it was also possible to demonstrate that similar principles pertained to early human T cell differentiation and that thymopoietin was active in these human systems [44, 46].

THYMOPOIETIN – IMMUNOREGULATION

Further studies with thymopoietin have revealed that its actions are not restricted to early T lineage cells. Rather, it appears to have complex actions on peripheral cells of the immune system and, in sum, it appears to have regulatory actions highlighted by the surprising finding that it can return the immune balance toward normal whether the initial immune deviation was in the direction of enhanced immune responsiveness or suppressed immune responsiveness [3, 52]. These principles are illustrated by the following experimental examples.

Adult Thymectomy in Mice

Adult thymectomy in mice is followed by progressive loss of the Ly 1,2,3 subset of splenic T cells. This is largely prevented by the administration of 1 μg TP-5 daily following thymectomy. This loss of Ly 1,2,3 T cells is accompanied by a functional loss of suppression, as measured by male skin graft rejection in female C3H mice (H-Y band rejection) [53]. Again, TP-5-treated mice largely maintained the state characteristic of thymus-intact mice and were tolerant of male skin containing the H-Y antigen. Furthermore, aged C3H female mice also lacked suppression for male skin, and this suppression was restored by TP-5 injections, which enabled older female mice to accept male skin grafts.

Generation of Cytotoxic Lymphocyte Precursor Units (CLP-U)

CLP-U can be evaluated in vitro in an allogeneic response. Pretreatment of the donor mice in vivo or the spleen cells in vitro does not affect CLP-U generated by optimal antigenic stimulation, but it does enhance CLP-U generated by suboptimal antigenic stimulation. Thus, in this model system, TP-5 does not act as an adjuvant but rather enhances the immune response when it is being suboptimally stimulated [54].

Development of Autoantibodies

Erythrocyte autoantibodies arise spontaneously in NZB mice or can be induced in other mouse strains by injection of cross-reacting rat erythrocytes [55, 56]. TP-5 enhances

the development of suppressor cells in both these systems and reduces the titer of autoantibodies but does not affect the development of antibodies to antigens exclusive to rat erythrocytes [75, 52].

Immunosuppression in Tumor-Bearing Mice

Mice bearing transplantable syngeneic tumors develop progressive immunosuppression, with demonstrable suppressor cells in their spleens [58]. TP-5 injections prevent and delay this immunosuppression and result in the development of cytotoxic cells, rather than suppressor cells, in the spleens of these animals. Concomitantly, tumor progression is reduced.

Antibody Response in Aged Mice

In aged mice there are impaired antibody responses to defined haptens, such as DNP (dinitrophenyl). These can be restored by infusion of young T cells or, most strikingly, by injection of TP-5 [39].

From these diverse examples it can be seen that thymopoietin acts in a seemingly different manner in different models of immune perturbation. For example, in autoantibody induction or in thymectomized mice, where the balance appears shifted toward lack of suppression, thymopoietin appears to restore suppressor activity. On the other hand, in tumor-bearing mice or in the antibody responses of aged mice, the balance appears shifted toward excess suppression and the effect of thymopoietin is to improve helper functions in these states [3, 52]. Perhaps these seeming incongruities can be explained on the basis of regulatory circuitry in the immune system, with thymopoietin acting to reestablish homoeostasis.

METABOLISM AND TOXICITY OF TP-5

TP-5 in human serum has a half-life of less than 30 seconds [47]. Yet analysis of its in vivo effects, using the CLP-U assay described above, has shown that the effects of a single injection of TP-5 persist for days to weeks. Apparently, as with induction of T cell differentiation (see above), only a brief pulse of action is required, and this suffices to trigger cellular changes with a longer persistence.

The breakdown of TP-5 is mediated by proteolytic enzymes in the serum, and it is rapidly reduced to its component amino acids [47]. This would suggest a low toxicity potential, and this has been borne out in actual testings. No toxicity has been obtained from doses up to 250 mg/kg, and subacute toxicity testing with three weekly injections in rats and dogs has revealed no toxicity from doses up to 10 mg/kg. Furthermore, approximately 100 human patients have been treated with doses up to 1 mg/kg for up to one year. No side effects whatsoever have been observed.

IMMUNOREGULATION IN HUMAN DISEASE

The preclinical studies with TP-5 suggesting low or absent toxicity and efficacy in restoring aberrant immunoregulation in a number of pathological models clearly carry important implications with respect to therapy in human disease [59]. Furthermore, the development of monoclonal antibodies defining functional subsets of human T cells raises the possibility of defining aberrant immunoregulatory states in human diseases, and the

potential for monitoring restoration of immune perturbations by TP-5. Clearly such studies are in their infancy, but already we have promising indications that such procedures may, in time, become reality.

Abnormal proportions of inducer and suppressor T cells, as measured by OKT series monoclonal antibodies, have already been recorded in infectious mononucleosis, multiple sclerosis and, most recently, rheumatoid arthritis [15, 59–62]. In rheumatoid arthritis, there is a decrease in $OKT8^+$ suppressor T cells, producing a high ratio of $OKT4^+/OKT8^+$. We already have preliminary indications that TP-5 can induce clinical remissions in rheumatoid arthritis. Furthermore, in preliminary studies, it appears that injections with TP-5 elevate the levels of suppressor cells in patients with rheumatoid arthritis and return the ratio of $OKT4^+/OKT8^+$ toward more normal levels. It is tempting to speculate that these observable changes in T cell subsets in rheumatoid arthritis are causally related to the pathogenesis of the disease and to the remission being induced by TP-5. The establishment that such clinical and diagnostic findings are real and related must await the completion of rigorous double-blind studies, which are being initiated. Similarly, the detection of immunoregulatory abnormalities in other disease groups, (as suggested by some of our experimental models) and their eventual treatment with TP-5, are further exciting prospects for the future.

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