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Reviews

Immunomodulating peptides

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Introduction

1985 marks the 60th anniversary of the definition by Gaston Ramon of 'adjuvants and immunity-stimulating substances' as products which, when used in combination with specific vaccines (in his case, diphteria or tetanus toxoid), enhance immunity levels above those that the vaccine is capable of eliciting when injected alone. Ramon found such an adjuvant activity in a wide variety of substances: agar, tapioca, starch oil, lecithin, aleurone seeds, even bread crumbs. In 1937, Jules Freund described the powerful adjuvant activity on both what we now call humoral and cell mediated immune reactions of an emulsion of killed tubercle bacilli in paraffin oil. The next step was to characterize the active principle of the tubercle bacilli. In France and Great Britain, the teams of Lederer, White and Jollès described the adjuvant activity of a peptidoglycolipid fraction (wax D) from human strains of *M. tuberculosis*. An active wax D contains a lipid part, mainly consisting of mycolic acids esterified to a polysaccharide (arabinogalactan), and also a nitrogen-containing moiety. The chemical structure of the latter was elucidated in 1968 by Migliore and Jollès[36]: it turned out to be closely related to the peptidoglycan which forms the backbone of mycobacterial and other bacterial cell walls. In the last decade, considerable progress was made, as we shall see, in the knowledge of glycopeptides and peptides of microbial origin endowed with immunostimulating activities. Concomitantly, significant activities on the immune system have been found for a great many peptides of animal origin. Some of these peptides stimulate certain functions of the immune system, others exert inhibitory activities; in addition, depending on the dose administered, the experimental conditions and the function which is under study, the same substance may exert either stimulating or inhibiting activities and the word 'immunomodulating' describes best this dual type of pharmacological effect. The purpose of this paper is to review present knowledge about peptides of relatively small mol. wt endowed with immunomodulating activities, excluding more complex polypeptides such as monokines (interferons) and lymphokines (interleukins).

Glycopeptides and peptides of microbial origin

Muramyl peptides

As stated above, attempts at characterizing the molecular entities responsible for the immunopotentiating activities of the mycobacterial cells present in Freund's adjuvant reached a turning point when water-soluble adjuvant-active fractions could be isolated from Mycobacteria. These studies culminated in 1974[16] in the identification of N-acetylmuramyl-L-alanyl-D-isoglutamine (muramyldipeptide, MDP, formula in fig. 1) as the minimal essential structure required for adjuvant activity. During the last ten years, muramylpeptides have been extensively studied from every possible viewpoint by many laboratories throughout the world. The whole field has been very lucidly reviewed recently by Adam and Lederer[2] and we shall simply summarize here the most salient data.

The cardinal feature of MDP and of the many active structural analogs which have been synthesized in the last ten years is their adjuvant activity on antibody production. With most antigens which were tested, muramylpeptides, when injected simultaneously and in the absence of mineral oil, exert an enhancing effect on antibody production which may equal that obtained with Freund's complete adjuvant. Such an adjuvant activity was demonstrated with complex vaccines (*Brucella abortus*, influenza virus, *Plasmodium falciparum* merozoites) and with chemically defined or synthetic vaccines (diphteria toxin octadecapeptide, hepatitis B virus surface antigen, luteinizing hormone-releasing hormone). Most significant results are obtained when MDP is covalently bound to the immunogenic peptide. MDP and several analogs are also capable of enhancing the nonspecific resistance of mice to various infectious agents (*Klebsiella pneumoniae, E. coli, Str. pneumoniae, Ps. aeruginosa, Candida albicans, Trypanosoma cruzi, Toxoplasma gondii*). In vitro, muramylpeptides activate macrophages to inhibit the growth of several tumor cell lines and/or to kill them.

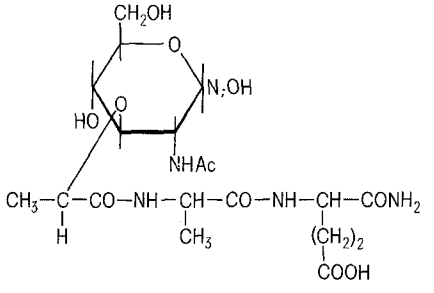

Figure 1. Structural formula of muramyldipeptide (MDP).

In vivo, some lipophilic derivatives of MDP (see below) as well as MDP itself when encapsulated in liposomes have shown activity in retarding the growth of transplanted tumors in the mouse, in preventing metastasis to distant organs and in increasing the survival time of the animals.

Structure-activity relationship studies in the muramylpeptide series indicated that L-alanine can be replaced, without loss of activity, by various L-amino acids, that the D-glutamic acid residue is essential and that the functionality of D-Glu is important. Many lipophilic derivatives of MDP, such as 6-O-stearoyl-MDP have been synthesized; they are generally more active than MDP in stimulating cell-mediated versus humoral immune responses; a lipophilic muramyltripeptide (MDP-L-Ala-phosphatidyl-ethanolamine, or: MTP-PE, fig. 2) enhances the resistance of mice to experimental virus infections[27]. One of the most interesting MDP analogs thus far prepared is a butyl ester named murabutide (N-acetyl-muramyl-L-alanyl-D-glutamyl-n-butyl ester, fig. 3), which is as active as the parent compound as an adjuvant of antibody production and enhancer of nonspecific resistance to infections, but is devoid of the pyrogenicity associated with MDP and other active analogs: murabutide does not elicit any febrile response even when injected by the i.c.v. route and does not stimulate the production of endogenous pyrogen[14].

It is noteworthy that the fundamental question of the mechanism(s) of the immunomodulating activities of the muramylpeptides remains by and large unanswered, but it is clear that the macrophages and the B lymphocytes are the main targets of such activities. There is no evidence for the presence of MDP-receptors at the surface of these cells. It is also noteworthy that when radiolabeled MDP is injected intravenously into mice, 95% of the compound is recovered unchanged within 2 h in the urine.

Intriguing connections between the neuroendocrine and the immune systems have been uncovered with respect to muramylpeptides. 'Sleep factor' S is a substance found in human cerebrospinal fluid and also in urine, which prolongs slow-wave sleep (SWS) when injected intracerebrally in rabbits, cats, rats and monkeys: as it turned out, acid hydrolysis of this factor yielded muramic acid, alanine, glutamic acid and diaminopimelic acid and, conversely, MDP was found to exert a similar somnogenic activity when injected, at higher doses, by the i.c.v. route in rabbits (for review, see Krueger et al.[30]). The nonpyrogenic murabutide is devoid of somnogenic activity but a dissociation between the pyrogenic and the somnogenic activities of MDP itself can be achieved through the use of antipyretics. Using a monoclonal antibody prepared against MDP, Chedid et al.[12,13] found that it was capable of binding to the SWS factor (thus confirming structural analogies between the latter and bacterial peptidoglycan fragments) and, in addition, that this antibody inhibited the lymphocyte-activating effect of a pyrogenic monokine elaborated by human monocytes upon in vitro stimulation with heat-killed staphylococci.

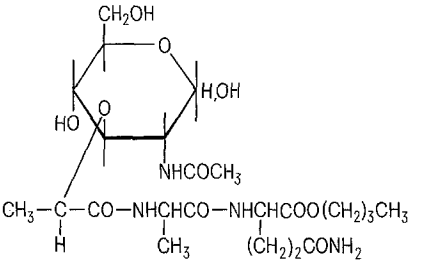

Figure 3. Structural formula of murabutide.

It thus appears that some of the factors produced by macrophages, such as the leukocytic pyrogen, a lymphocyte-activating factor and the SWS factor are intimately related to MDP, that is to the structural unit of the bacterial peptidoglycans which the macrophages are loaded with as a consequence of their scavenger role. Already in 1976, Jollès[25] formulated the hypothesis that fragments of bacterial peptidoglycans, liberated in the gut through the action of lysozyme, behaved as 'natural immunostimulants', helping the host to resist microbial infections. Pushing this view a little further, Chedid et al.[13] and Adam and Lederer[2] propose that peptidoglycan derivatives such as MDP be considered as a new class of vitamins, i.e. exogenous substances which cannot be synthesized by the host but which are required for some essential physiological functions, such as resistance to pathogens and slow wave sleep. The use of synthetic MDP and analogs as vaccine adjuvants and enhancers of antimicrobial resistance may be a new example of the pharmacological applications of vitamins and of their synthetic analogs.

Lipopeptides

For a while, the presence of a muramic acid moiety was considered to be essential for the immunopotentiating activities of peptidoglycan fragments from bacterial cell walls. This 'dogma' was refuted when our group demonstrated in 1979 that chemical coupling between lauric acid and a biologically inactive tetrapeptide, L-Ala-D-Glu[LL,A_2pm(Gly)]NH_2 (which had been isolated from

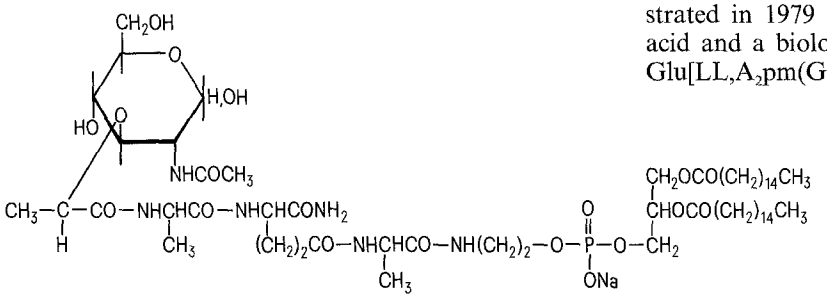

Figure 2. Structural formula of MDP-L Ala-phosphatidyl-ethanolamine (MTP-PE).

immunostimulating crude extracts of a Streptomyces strain) yielded a compound endowed with marked in vitro and in vivo immunopotentiating activities[34,35,61]. This lauroyltetrapeptide was then prepared by total synthesis (formula in fig. 4), allowing a thorough study of its biological properties which will be summarized hereafter. The lauroyl tetrapeptide (RP 40639, pimelautide: proposed international denomination) stimulates in vitro the phagocytic and bactericidal activities of mouse macrophages and of human blood monocytes and, in the mouse, enhances the clearing activity of the reticuloendothelial system. Administered by parenteral routes to the mouse, it exerts an accelerating effect on the recruitment of prethymic lymphocytes from the bone marrow to the spleen, enhances the induction of cytolytic lymphocytes against allogenic tumor cells and stimulates natural killer (NK) cell activity in the spleen and the peripheral blood. These various activities indicate that the lauroyltetrapeptide is able to interact (directly or indirectly) with cells of the mononuclear-phagocyte system, with T lymphocytes and with NK cells. It has also been shown that, in vitro, the lauroyltetrapeptide enhances the helper activity of human blood T lymphocytes for the differentiation of B lymphocytes into plasmocytes.

In the mouse, the compound exerts an adjuvant effect on delayed-type hypersensitivity (DTH) reactions and on antibody (Ab) production against various antigens: globally speaking and in this respect, lauroyltetrapeptide appears to be more active than the muramyl dipeptides as an adjuvant of DTH reactions and less active than the former as an adjuvant of Ab production. The lauroyltetrapeptide enhances the resistance of mice to microbial infections *(Klebsiella pneumoniae, Listeria monocytogenes)* and inhibits the production of pulmonary metastases in mice inoculated intravenously with B16 melanoma cells or intradermally with Lewis lung carcinoma cells. Administered in association with suboptimal doses of cyclophosphamide, the compound enhances the activity of this anticancer drug on L 1210 leukemia and Lewis lung carcinoma. In all these in vivo systems, the lauroyltetrapeptide was active at parenteral doses ranging between 0.1 and 3 mg/kg, whereas its acute LD_{50} in the mouse by the i.v. route is 410 mg/kg. Eighty close or more distant analogs of the lauroyltetrapeptide have been synthesized and studied in the Rhône-Poulenc laboratories: it was shown that diaminopimelic acid could be replaced by lanthionine without loss of activity and that lipodipeptides (such as palmitoyl-L-Ala-D-glutamic acid or behenoyl-L-Ala-D-glutamic acid) still retained some immunopotentiating activity in vivo.

The interesting properties of lipopeptides were amply confirmed in other laboratories; at the Pasteur Institute in Paris, an enhancing activity on resistance to microbial infections was found for a 'desmuramyl peptidolipid' such as 1,0-(L-Ala-D-isoGln-L-Ala)-glycerol-3-mycolate[44] and, in 1981, the Fujisawa Research Laboratories in Osaka[21] reported the immunopotentiating activities of compound FK-156, namely: D-lactoyl-L-alanyl-γ-D-glutamyl-(L)-meso-2,6-diaminopimeloyl-glycine, which is a metabolite of *Streptomyces olivaceogriseus*. A great many analogs of FK-156 were synthesized and studied in these laboratories, one of the most active being the lipopeptide FK-565 (formula in fig. 5). This compound, as well as several others, was shown to enhance the resistance of normal or immunocompromised mice to bacterial and fungal infections, and to exert immunotherapeutic activities in mice grafted with syngeneic tumor cells[22,38]. Results obtained with the FK-156 analogs showed that the D-configuration of glutamic acid and the L-configuration of diaminopimelic acid, at the site combining with glutamic acid and glycine, are essential for biological activity, and also established that N^2-(γ-D-glutamyl)-2(L),2'(D) diaminopimelic acid was the minimal structural unit capable of exerting the immunopotentiating activities of this series of compounds[57].

Recently, a novel synthetic compound, in which the L-alanyl-D-isoglutamine residue of muramyldipeptide was linked to 1-aminoadamantane (adamantylamide dipeptide) was shown to exert an adjuvant effect on a DTH reaction in guinea pigs and to stimulate labeled thymidine incorporation in the liver, thymus and spleen of the animals[32].

The main cellular targets of the immunomodulating lipopeptides described above are the phagocytic cells and, directly or indirectly, the T lymphocytes. Little if any direct stimulation of B lymphocytes can be evidenced. On the other hand, the lipoprotein from the outer membrane of *Escherichia coli* is known to be a potent mitogen of B lymphocytes from various animal species. This lipoprotein is composed of 58 amino acids and contains at the N-terminal end one amide-linked and two ester-linked fatty acids bound to glyceryl cysteine, as shown in 1975 by Braun[11]. The molecular structure responsible for the B-cell mitogenicity resides in its fatty acid-containing N-terminal region: to identify the minimal molecular structure of the lipoprotein and of active lipopeptide fragments responsible for mitogenicity, Bessler et al.[8] synthesized the N-terminal pentapeptide part of the lipoprotein, which contains three fatty acids, and tested its in vitro activity (in spleen cells from C3H/HeJ mice). The synthetic compound (fig. 6) turned out to be as active as a mitogen and adjuvant of antibody production against sheep erythrocytes as the whole lipoprotein. In further work Bessler et al.[7] reported the potent B-lymphocyte stimulating activity of a still shorter synthetic compound, namely N-palmitoyl-(S)-seryl-(S)-(S)-asparaginyl-(S)-alanine (palmitoyl-tetrapeptide). It will be of interest to

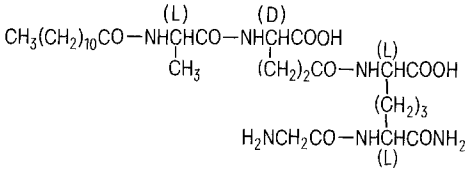

Figure 4. Structural formula of pimelautide, a lauroyltetrapeptide.

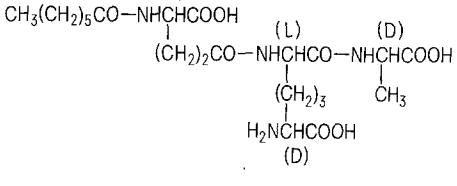

Figure 5. Structural formula of compound FK-565.

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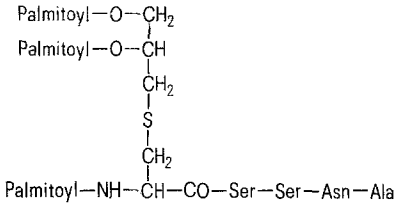

Figure 6. Structural formula of s synthetic lipopeptide analogous to the N-terminal sequence of *E. coli* lipoprotein.

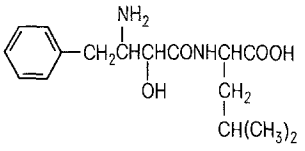

Figure 8. Structural formula of bestatin.

know the in vivo immunomodulating activities of these synthetic lipopeptides.

In conclusion, peptides lacking the muramic acid moiety are capable of exerting immunostimulating activities as significant as those of the muramylpeptides, although the nature and spectrum of these activities are not strictly identical with the latter. Lipopeptides, in particular, such as the lauroyltetrapeptide (RP 40 639) and FK-565, appear to be promising candidates for clinical evaluation of their therapeutic usefulness, especially in situations in which the immune system of the patient needs reinforcement.

Ciclosporine

Ciclosporine (formerly called cyclosporin A) is a cyclic undecapeptide isolated at the Sandoz Research Laboratories from the fungus *Tolypocladium inflatum Gams* (structure in fig. 7), which is highly effective in inhibiting both humoral and cell-mediated immune responses[10]. Its suppressive activity is exerted specifically on T lymphocytes and it is reversible. Ciclosporine acts at an early stage of antigenic triggering of the T lymphocytes and this activity is mediated, at least in part, by inhibition of lymphokine secretion by T cells, which provides growth and differentiation signals for T and B lymphocytes and for macrophages. It has been shown that ciclosporine inhibits interleukin 2 gene expression in T lymphocytes at the level of mRNA transcription[29]. In vivo, in various species, ciclosporine is effective in preventing the rejection of allogenic organ transplants, and it has been successfully used in humans in bone marrow and kidney transplantation. Indeed, in view of the selectivity of its action, and the favorable dose ratio between toxic and pharmacological effects, ciclosporine is the prototype of a new generation of immunosuppressive drugs, far superior to those previously used in human medicine, such as azathioprine or cyclophosphamide. In addition, ciclosporine is effective in various animal models of auto-immune disorders (auto-immune uveitis in the rat, murine lupus-like disease in NZB/NZW mice, adjuvant arthritis in rats) and clinical studies have yielded promising results in the treatment of juvenile onset insulin-dependent diabetes.

Another cyclic peptide, a hexacyclodepsipeptide named cyclomunine, has been isolated from the fermentation broth of the fungus *Fusarium equisitii,* at the Servier laboratories. In vitro, cyclomunine was shown[41] to inhibit the responses of human lymphocytes to mitogens, soluble antigens and allogenic cells and the proliferation of lymphoblastoid cell lines.

Bestatin

Some years ago, Umezawa[59,60] formulated the hypothesis that screening for compounds able to bind to the surfaces of cells of the immune system might result in the finding of immunomodulating substances. Since aminopeptidases, alkaline phosphatase and esterase are located on the cell surface of macrophages and lymphocytes, a search was begun in fermentation broths for inhibitors of these enzymes. This led, among others, to the discovery of bestatin (produced by *Streptomyces olivoreticuli*), an inhibitor of leucine aminopeptidase, which contains a single peptide bond between L-leucine and 2-hydroxy-3-amino-4-phenylbutyric acid (fig. 8). The immunomodulating activities of bestatin have been extensively studied[59,60]; the compound stimulates cell-mediated immunity over a wide range of doses and, at the higher doses, enhances antibody production. It is claimed to induce IL-1 production by macrophages and to augment IL-2 production by mitogen-stimulated lymphocytes; its antitumor effects in murine models seem to be exerted through activation of cytolytic T lymphocytes and natural killer (NK) cells. Clinically, bestatin is used in several

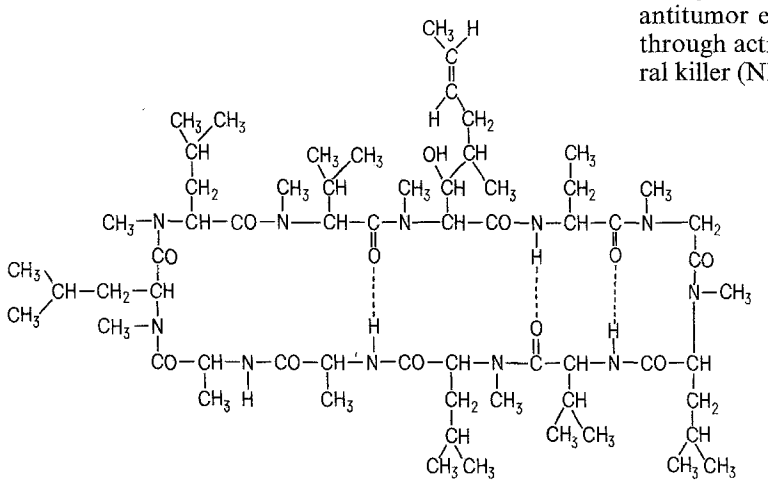

Figure 7. Structural formula of ciclosporine.

centers as an 'immuno-restoring agent' mostly in cancer patients: as with all agents evaluated in such a context, it is still too early to draw conclusions about overall efficacy.

Peptides of animal origin

Thymic hormones

The modern era of thymology began in 1961 when Jacques Miller[37] showed that neonatal thymectomy in mice results in failure of lymphoid tissue development and of the maturation of several parameters of immunological competence. The thymus gland is essential to the development of the various subsets of T lymphocytes. The endocrine function of the thymus, i.e. the secretion of hormones by the epithelial compartment of the gland, has been the subject of much work since the pioneer research of Abraham White in 1966[19], and we are now confronted with six different peptides or polypeptides which were isolated from crude thymic extracts or from serum and which exert hormonal modulating activities mostly restricted to pro-thymocytes or to T lymphocytes. It is also important to note that other immunomodulating polypeptides, such as interleukins 1 and 2, are elaborated in the lymphoid compartment of the thymus. Several reviews have been devoted recently to thymic hormones[5,6,31], which can be referred to for a more comprehensive treatment of this subject. At the present stage, 3 thymic hormones are fully characterized, which in all likelihood are produced in the thymus and which have been shown to induce specific T cell-markers precursor cells and to promote T lymphocyte functions: those are thymopoietin (49 amino acids), thymosin $\alpha 1$ (28 amino acids) and thymulin (a Zn-containing nonapeptide). Their amino acid sequences are given in figure 9; as can be seen there is no homology between the amino acid sequences of these peptides. Two others should also be mentioned; the thymic humoral factor (THF), a polypeptide of mol. wt 3200 which has not been sequenced yet, and TP-5, a pentapeptide corresponding to amino acid sequence 32 to 36 of thymopoietin (Arg-Lys-Asp-Val-Tyr) and which exerts the same biological activities as thymopoietin. Other peptides have been extracted from the thymus, such as thymosin α_7, α_{11} (35 residues), β_4 (43 residues), β_8 (39 residues), β_9 (41 residues), β_{10} (42 residues) but, except for β_4, little is known about their biological activities.

To take one example of the biological profile and possible biomedical applications of thymic hormones, thymulin, as stated by J.F. Bach[4] promotes most T cell functions when it is injected into adequate recipients. According to the immune status of the recipient, thymulin enhances, inhibits or leaves unchanged the function which is under study. For instance, thymulin inhibits the generation of cytotoxic T lymphocytes (against allogenic cells) in normal mice but stimulates it in adult-thymectomized mice infraoptimally immunized with the allogenic cells; it is without effect if the thymectomized mice are immunized with an optimal number of allogenic cells. Thymulin enhances delayed-type hypersensitivity reactions to dinitrofluorobenzene in adult-thymectomized mice but depresses it in normal mice. The most probable explanation of such seemingly paradoxical facts is that the hormone is able to stimulate both helper and suppressor T lymphocytes: depending on the dose administered and the precise immune status of the animal, one effect is preferentially expressed or the two effects counteract each other.

As evident from the prevailing T cell selectivity of the thymic hormones, it is in the management of illnesses associated with dysfunctions of the various T cell populations that thymic hormones may find their greatest usefulness. Many clinical studies have been performed in the recent past, often with encouraging results but in a limited number of applications, with relatively crude thymic extracts. Phase I and II clinical studies are now in progress with synthetic thymic hormones (thymosin α_1, thymopoietin and TP-5, thymulin) and with THF, in several pathological conditions in which abnormal T cell functions are present or suspected: severe viral infections in immunocompromised patients, lung cancer, head and neck cancer, rheumatoid arthritis, ataxia telangectasia, Di George's syndrome. It appears to be too early to draw conclusions about the therapeutic future of the various thymic hormones, but it is already clear that the pure chemically defined thymic peptides are as active as the crude thymic extracts.

Some thymic peptides, such as thymosin α_1 and β_4, have been detected in the central nervous system (CNS) and may well participate in neuroendocrine functions; Renoux[53] has come forward with the hypothesis that the thymus is a part of a hypothalamo-hypophyseal-thymic axis controlling production of hormones and transmitters which regulate the immune system and the connections of the latter with the CNS.

Synthetic analogs of some thymic hormones have been prepared and studied for biological activity; we have already mentioned TP-5, a pentapeptide which is as active as thymopoietin in some tests. Many thymulin analogs have been synthesized[4], some of which exert antagonistic action versus the native molecule. It appears that the minimal sequence necessary for binding to the thymulin receptor is Lys-Ser-Glu-Gly-Gly. Some of the active peptides (thymulin agonists), such as: (Har[3]) thymulin and (D-Asn[9]) thymulin possess in vivo a longer half-life than thymulin itself, which makes them potential candidates for clinical evaluation.

Tuftsin

In 1967, Victor A. Najjar and his coworkers at Tufts University, in Boston, demonstrated the existence of a leukophilic immunoglobin of the G class (IgG), called leukokinin, which binds to blood granulocytes and monocytes and to tissue macrophages and stimulates their phagocytic activity. Shortly afterwards, the same authors showed that a tetrapeptide, L-Thr-L-Lys-L-Pro-L-Arg, present in the Fc region of leukokinin and corresponding to residues 289–292 of its heavy chain, was indeed responsible for the phagocytosis-stimulating activities of leukokinin; they called it tuftsin. Tuftsin requires two enzymes for its in vivo liberation in active form from the parent carrier IgG: tuftsin-endopeptidase (in the spleen) cleaves distal to the arginine end and leukokininase, present on the outer side of the plasma membrane of phagocytic cells, cleaves proximal to the threonine residue. In vivo, tuftsin stimulates all functions of phagocytic

cells: pinocytosis, phagocytosis, motility, antigen processing, bactericidal and tumoricidal activities. Administered in vivo, tuftsin modulates humoral and cell-mediated immune functions; as with other immunomodulating agents, dosage and time of administration are critical parameters. Some of these immunomodulating effects may be observed up to two weeks after administration of the peptide, indicating that its binding to cell receptors may initiate a cascade of reactions resulting from its primary interaction with phagocytic cells. Among other in vivo effects, tuftsin was shown to enhance natural cytotoxicity against tumor cell targets, mediated by macrophages and monocytes as well as by NK cells. In the mouse, tuftsin was shown at minute doses to enhance resistance against experimental microbial infections and to delay mortality in several tumor transplantation models, such as L 1210 leukemia and B16 melanoma. Administered repeatedly, tuftsin exerted a restorative activity on some immunodepressed functions of aged mice[40].

The fact that tuftsin is a physiological activator of macrophage functions can be inferred from the observations of congenital and acquired tuftsin deficiency states in humans exhibiting recurrent severe infections. Preliminary clinical studies are under way with tuftsin; a phase 1 trial in advanced cancer patients has shown no toxic effects of doses up to 1 mg/kg i.v.; lower doses caused an increase in the total count of peripheral blood leukocytes and in mononuclear cell cytotoxicity.

Several analogs of tuftsin have been synthesized and studied for their biological activities, in various laboratories, for example a decapeptide (Thr-Ile-Ser-Lys-Ala-Lys-Gly-Gln-Pro-Arg) and a tuftsinyltuftsin-octapeptide (Thr-Lys-Pro-Arg-Thr-Lys-Pro-Arg). Finally, in view of the increasing awareness of mutual interactions between the immune and the neuroendocrine system, it is noteworthy that neuropeptides such as substance P, neurotensin and kentsin (Thr-Pro-Arg-Lys) compete with tuftsin for its binding sites on phagocytic cells and that i.c.v. injection of tuftsin into rats exerts an analgesic effect. For further details about the various activities of tuftsin the reader is referred to the proceedings of a symposium held in 1983 at the New York Academy of Sciences[40].

Peptidic inhibitors of the in vitro activity of tuftsin, closely related structurally to the latter, have been described, for example the Lys-Pro-Arg tripeptide or the Thr-Lys-Pro-Pro-Arg pentapeptide. But it appears to be of greater interest that another tripeptide, namely Thr-Lys-Pro, while not exerting any antagonistic effect on tuftsin activity, does directly inhibit various macrophage functions, i.e. exerts activities which are opposite to those of tuftsin (Thr-Lys-Pro-Arg). André Capron and coworkers[3] have shown that IgG molecules, when they bind to membranes of *Schistosoma mansoni* larvae, are cleaved by proteolytic enzymes secreted by the parasite and that released peptidic fragments exert inhibitory effects on macrophages: such an activity can be ascribed to the tripeptide mentioned above (TKP). These authors have shown that inhibition of β-glucuronidase release, production of interleukin 1 and of superoxide anion occurs when rat peritoneal macrophages are preincubated with TKP. Chemotaxis and IgE-specific receptor expression are inhibited in rat peritoneal macrophages and in human alveolar macrophages after in vitro treatment with TKP, at concentrations which do not affect cell viability.

In conclusion and from a pharmacological viewpoint, two peptides of closely related chemical structure, tuftsin (Thr-Lys-Pro-Arg) and TKP (Thr-Lys-Pro) can be considered as an immunostimulating agent, for the first one, and as a potential anti-inflammatory and anti-allergic agent for the second.

Thymosin α_1

Ac-Ser-Asp-Ala-Ala-Val-Asp-Thr-Ser-Ser-Glu-Ile-Thr-Thr-Lys-Asp-Leu-Lys-Glu-Lys-Lys-Glu-Val-Val-Glu-Glu-Ala-Glu-Asn-OH

Thymopoietin II

H-Pro-Glu-Phe-Leu-Glu-Asp-Pro-Ser-Val-Leu-Thr-Lys-Glu-Lys-Leu-Lys-Ser-Glu-Leu-Val-Ala-Asn-Asn-Val-Thr-Leu-Pro-Ala-Gly-Glu-Gln-Arg-Lys-Asp-Val-Tyr-Val-Glu-Leu-Tyr-Leu-Gln-Ser-Leu-Thr-Ala-Leu-Lys-Arg-OH

Thymulin

Glu-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn-OH

Figure 9. Structural formulae of three peptidic thymic hormones.

Peptides derived from fibrinogen

Biochemical analysis of some pathological situations has hinted at the existence of endogenous peptides endowed with immunomodulating activities. For instance, Girmann et al.[18] hypothesized that there was a correlation between the impairment of cell mediated immunity in advanced cancer disease and the frequent presence in the latter of circulating fibrinogen degradation products (small peptides released by plasmin and called micromolecular fibrinogen degradation products, FDP). Indeed, terminal FDP obtained by digestion of human fibrinogen with human plasmin inhibited in vitro, in a dose-dependent manner, PHA-induced transformation of lymphocytes and, injected i.v. at 130–160 µg/mouse 24 h after immunization with sheep red blood cells (SRBC), inhibited the production of plaque forming cells (secreting antibodies against SRBC) in the animals' spleens. The mol. wt of the active peptides was estimated to be around 5000.

These observations were confirmed by Plow et al.[52]: at concentrations of 150 µg/culture, fibrinogen-derived peptides profoundly inhibit basal and PHA-stimulated protein synthesis by human peripheral blood lymphocytes; this inhibition (not seen with the parent molecule) is observed when the cells are exposed for at least 4 h to the peptides, and is not corrected by removal of the peptides. The latter do not inhibit binding of PHA to the cells and do not dissociate cell-bound mitogen. Molecular exclusion chromatography on Biogel P-4 showed that only one fraction, probably containing 3–5 peptides, was active. Further studies by the same group[15] showed that the active peptides inhibit PHA, PWM and allogenic cell-stimulated lymphocyte proliferation, B and T cells appearing equally susceptible. Human peptides inhibit the response of murine spleen cells to mitogens; thymidine uptake by two continuous lymphoblastoid cell lines is also inhibited by the peptides, whereas they stimulate thymidine uptake by a diploid fibroblast cell line.

Kopec et al.[28] studied the effect of peptides (mol. wt > 3500) separated by dialysis from a plasmin digest of human fibrinogen on delayed skin hypersensitivity reactions to oxazolone in mice, and noted a strong inhibition of these reactions when 200 µg were injected s.c. in the area drained by the same lymph nodes as the site of sensitization. When the peptides were injected in the site of tumor implantation in mice grafted with a transplantable sarcoma, these authors observed an acceleration of tumor growth and an increase in the number of pulmonary metastases.

Gerdin et al.[17] have shown that two fibrinogen-derived peptides of defined structure (Ala-Arg-Pro-Ala-Lys and Thr-Ser-Glu-Val-Lys) endowed with vasoactive properties (enhancement of microvascular permeability) suppress in vitro the responses of murine spleen lymphocytes to LPS and Con A. The activities of these two vasoactive peptides appear to be too low, however, to account for the total activity seen with plasmin digests of fibrinogen. It is noteworthy that, as shown by Kopec et al.[28], the first pentapeptide, namely Ala-Arg-Pro-Ala-Lys, was as active as tuftsin in stimulating phagocytosis of *S. aureus* by mouse peritoneal macrophages, while the second was without effect.

Peptide fragments of β_2-microglobulin

We have here another example of the lessons to be learned from immunopathological situations. It is known that cell mediated immune functions are often impaired in uremic patients. In 1979, Abiko et al.[1] reported the presence in the hemodialysates from such patients of a small peptide which, in vitro, at relatively high concentrations (3–5 mg/ml) inhibited the formation by human peripheral blood lymphocytes of rosettes with sheep red blood cells. This inhibitor was sequenced and it turned out to be the heptapeptide His-Pro-Ala-Glu-Asn-Gly-Lys, which is identical to segment 13–19 of β_2-microglobulin, a protein present on the cell surfaces of many species as part of the major histocompatibility antigen complex and which is normally metabolized in the kidneys (hence the increased plasma concentration in patients with renal failure). Rola-Pleszczynski et al.[54] studied the effect of this heptapeptide on the cytotoxicity of human peripheral blood lymphocytes for herpes simplex virus-infected target cells. They found that low concentrations (10^{-7} M) of the peptide, present during the 5-h cytotoxicity assay (release of radioactivity from ^{51}Cr-labeled target cells) enhanced the cytotoxicity of the lymphocytes, whereas higher concentrations (10^{-5} M or more) were inhibitory. Furthermore, when the peptide was added to a 72-h sensitization phase, in which lymphocytes and target cells were cocultured, the subsequent cytotoxic activity of the lymphocytes was greatly enhanced in the presence of 10^{-9} M to 10^{-7} M concentrations but unchanged in the presence of higher concentrations. The parent molecule, β_2-microglobulin, was pratically without effect in this test. Analogs of the heptapeptide were synthesized and studied: the des-His-hexapeptide had little enhancing effect on fresh lymphocyte cytotoxicity but it enhanced cytotoxic activity following presensitization.

Met-Enkephalin
Tyr-Gly-Gly-Phe-Met
Leu-Enkephalin
Tyr-Gly-Gly-Phe-Leu
Substance P
Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
β-Endorphin
Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Val-Lys-Asn-Ala-His-Lys-Lys-Gly-Gln
Angiotensin II
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-NH_2
Vasoactive intestinal peptide
His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH_2
Somatostatin
Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys

Figure 10. Structural formulae of neuropeptides for which possible immunomodulating activities have been reported.

Peptides from colostrum and from milk

In the course of their studies on ovine colostral immunoglobulins, Janusz et al.[23] found that the IgG2 isolated by chromatography on DEAE-cellulose were contaminated with a proline-rich polypeptide (PRP) of mol. wt estimated to be 6000 which was able to increase the permeability of skin vessels and, in addition, exerted immunomodulating activities, as shown by Wieczorek et al. [62]. By digestion of PRP with chymotrypsin, Staroscik et al.[56] obtained a nonapeptide (mol. wt 1000) which showed a biological activity similar to that of PRP. These authors determined the amino acid sequence of this nonapeptide which is: Val-Glu-Ser-Tyr-Val-Pro-Leu-Phe-Pro. Like PRP the nonapeptide stimulates low and inhibits high antibody responses of mice against sheep erythrocytes; it stimulates the maturation of thymocytes and the generation of suppressor T cells. Altogether, these results suggest that PRP and the nonapeptide (which, in the comparative tests that were performed, was active at doses similar to those of PRP but, on a molecular basis, was about six-fold less active than PRP) interact with T-cell precursors, inducing both T-suppressor and T-helper cells, the net effect depending on the immune status of the animals. Staroscik et al.[56] stress that the effects of PRP and the nonapeptide resemble, in several respects, those of the thymic polypeptides and point to a low degree of amino acid sequence analogy with some of the β-thymosins.

Five years ago, our research team became interested in the possibility that immunomodulating peptides could be extracted from human casein. Our reasoning was that, long before the advent of effective antimicrobial therapy, man competed with some success against various infectious aggressions: he was probably submitted to a sort of natural immunostimulation through contact with a variety of microorganisms and bacterial substances or exposed to immunostimulants derived from his food. Since mother's milk is usually man's first food, we decided to prepare enzymatic fragments of human casein and to study their possible immunomodulating activities in two in vitro models: a) secretion of hemolytic antibodies by spleen cells from mice which had been immunized in vivo with sheep red blood cells (SRBC); b) phagocytosis of opsonized SRBC by resident peritoneal mouse macrophages. Delipidated human casein was digested with trypsin and the enzymatic digest was fractionated on Sephadex G-50: it was found that peptidic fractions cor-

responding to mol. wt in the range of 2000 to 600 were active (stimulation) in both tests[26]. Further purification of the active fractions was performed by chromatography on DEAE-Sephadex A-25, Sephadex G-15, Dowex 50 × 4 and finally by reverse-phase HPLC. A constant stimulating activity on phagocytosis was observed throughout these purification steps, whereas the stimulating effect on antibody secretion vanished in the course of purification.

A pure peptide was obtained, which exerted a marked in vitro stimulation of phagocytosis at a concentration as low as 0.1 μM, and which significantly increased the survival time of mice when administered intravenously at 0.3 and 1 mg/kg, 24 h before i.v. inoculation of a lethal dose of *Klebsiella pneumoniae*. The pure peptide was sequenced and its structure found to be: Val-Glu-Pro-Ile-Pro-Tyr; it was then prepared by total chemical synthesis and the in vitro and in vivo stimulating activities were confirmed with the synthetic hexapeptide[45]. Furthermore a number of tripeptides, such as Gly-Leu-Phe, were found to possess significant stimulating activities on phagocytosis by mouse macrophages[46].

Neuropeptides

In spite of an abundance of anecdotal evidence suggesting that mental stress, bereavement or depression may adversely affect the resistance of the human organism to infections and possibly to malignancies, in the recent past the immune system was still considered to be completely autoregulated through its own cells and soluble mediators. Within the last ten years, however, a number of anatomical, physiological, neurochemical and psychological data have demonstrated that there exists a vital link between the immune system and the central nervous system (CNS) and that these two systems 'communicate with each other'. Neuroendocrine hormones of a peptide nature seem to play an essential role as signals from the CNS to the immune system. As stated by Blalock and Smith[9], 'there is suggestive evidence for the existence of a complete regulatory loop through common or related peptide hormones and receptors, between the immune and neuroendocrine systems'. Inasmuch as there is still some controversy about the intrinsic functions of most neuropeptides as a new class of neurotransmitters, their interaction with lymphoid cells is an important fact.

A nonexhaustive search of the literature revealed that evidence for an interaction with the immune system has been reported for the following neuropeptides: endorphins, enkephalins, angiotensin, substance P, somatostatin and the intestinal vasoactive peptide (VIP). Amino acid sequences of these peptides are given in figure 10. One must stress that so far the great majority of the experiments showing links between these peptides and the lymphoid cells have been performed in vitro.

In 1979, Wybran et al.[64] reported the presence of (Met)-enkephalin- as well as morphine-like receptors on human peripheral blood lymphocytes and, using the highly sensitive active T-rosette assay, showed that (Met)-enkephalin enhanced the percentage of such active rosette-forming cells in vitro, whereas morphine decreased it; these effects were reversed by the morphine antagonist naloxone. The same year, Hazum et al.[20] reported the presence of β-endorphin receptors on human lymphoblastoid cell lines. Later McCain et al.[33] described the inhibition of PHA-induced blastogenesis of human lymphocytes by β-endorphin and suggested that this effect operated via non-opiate receptor mechanisms, since it was not reversed by naloxone. Johnson et al.[24] reported that treatment of mouse spleen cells with inducers of interferon α resulted in the production by these cells of corticotropin (ACTH)- and endorphin-like activities; both ACTH and α-endorphin turned out to be potent inhibitors of the in vitro antibody response to a T-cell dependent antigen (sheep erythrocytes), whereas (Met)- and (Leu)-enkephalins as well as β-endorphin were moderately active as inhibitors. More recently, Wybran[63] and Plotnikoff et al.[51] reported that in vitro, at concentrations ranging between 10^{-5} and 10^{-8} M, (Leu)-enkephalin and (Met)-enkephalin increased the natural killer (NK) activity of human peripheral blood mononuclear cells (against K-562 target cells) by about 30%. This stimulation was abolished by incubation of the effector cells with equimolar naloxone.

Substance P, which among the neuropeptides may be one of the best candidates for the status of neurotransmitter (transmission of painful sensation from peripheral receptors and CNS?), and which has been identified in the central and peripheral nervous systems and in the intestinal tract, was shown by Payan et al.[47] to stimulate human T-lymphocyte proliferation at concentrations as low as 10^{-10} M; the same authors[48] described the existence of stereospecific receptors for substance P on a line of human lymphoblasts. Furthermore, in subsequent studies[49], they showed binding of labeled substance P to 21% of human peripheral blood T-lymphocytes before PHA stimulation of the latter and to 35% after PHA stimulation. Interestingly, the suppressor-cytotoxic and the helper-inducer subsets (identified with monoclonal antibodies) contained substance P-reactive cells at respective mean frequencies of 10% and 18%, thus showing some discrimination between these two subsets with respect to their affinity for substance P. Human B lymphocytes, monocytes, polymorphonuclear leukocytes and platelets, on the other hand, showed only minimal specific binding of substance P. O'Dorisio et al.[42] have reported that human peripheral blood lymphocytes possess functional receptors for the vasoactive intestinal polypeptide (VIP), as shown by a potent stimulation of adenylate cyclase activity. On the other hand, VIP did not stimulate the adenylate cyclase activity of neutrophils or monocytes. More recently, Ottaway and Greenberg[43] investigated the binding of radioiodinated VIP to lymphocytes isolated from mouse lymph nodes and thymus: VIP-binding sites were found predominantly on T cells and, in the presence of VIP, the in vitro responses of the cells to concanavalin A and PHA was inhibited in a dose-dependent manner, whereas their response to a B-cell mitogen (lipopolysaccharide) was unchanged.

Somatostatin is a tetradecapeptide, initially isolated from the hypothalamus and shown to be a potent inhibitor of the secretion of growth hormone; this peptide, which is localized in neural and gastro-intestinal tissues, can also inhibit secretion from a wide array of endocrine and exocrine glands. Payan et al.[50] have shown that, at concentrations from 10^{-13} to 10^{-9} M, somatostatin inhibits the proliferation of PHA-stimulated T lymphocytes, purified by rosetting from human peripheral blood. This

inhibition is associated with a mean degradation of 95% of somatostatin following 24 h incubation at 37 °C.

Finally, with respect to the octapeptide angiotensin II, Thomas and Hoffman[58] have shown that macrophages from guinea pig peritoneal exudate express specific receptors for this substance, receptors which are responsible for most of the uptake of angiotensin II by macrophages; binding of angiotensin II to receptors correlates with pulsing for angiotensin II-specific T cell stimulation in the immune response against this peptide. These authors speculate that one possible role for macrophage angiotensin II-receptors may be in the physiological maintenance of vascular fluid pressure: activated macrophages are known to secrete the angiotensin-converting enzyme (ACE) which, by proteolysis, converts angiotensin I into angiotensin II; since this reaction contributes to the maintenance of blood pressure, macrophages may play a role there through the secretion of ACE. Shimoda and Yazaki[55], on the other hand, have found angiotensin II binding by human mononuclear leukocytes but not by granulocytes, erythrocytes and platelets.

In conclusion, specific receptors for a wide variety of neuropeptides have been demonstrated in several cell populations of the immune system. In most instances, these receptors have been shown to be functional, which clearly indicates that neuropeptides may indeed modulate (inhibit or stimulate) the activities of immune cells. Neuropeptides are thus obvious candidates as natural signals between the neuroendocrine system and the immune system. As stated previously, much remains to be done in applying this knowledge to the situations which exist in vivo.

Another potentially fruitful area of research is represented by the interaction with the neuroendocrine system of peptides first described as natural immunomodulating agents. We have already mentioned the somnogenic activity of certain muramylpeptides and the fact that some thymic peptides have been detected in the CNS. It is also known that substance P competes with tuftsin for its binding sites on phagocytic cells and that i.c.v. injection of tuftsin produces an analgesic effect in rats. Recently Mitsuma et al.[39] have provided experimental evidence suggesting that tuftsin acts at the hypothalamus level to stimulate in rats the release of the thyrotropin-releasing hormone.

Conclusion

We have attempted to review present evidence that several classes of peptides exert a powerful influence on the immune system and therefore deserve a place of honor among immunomodulating agents. Many immunomodulating peptides are directly derived from the microbial world, like muramyldipeptide (MDP), a basic unit of the bacterial cell wall peptidoglycan capable of exerting an immunoadjuvant effect, or like ciclosporine, a fungal metabolite which has turned out to be a remarkably selective immunosuppressive agent. The development as immunomodulators of the lipopeptides as well as of many MDP derivatives results from a semi-synthetic approach based on the structures of microbial peptides. On the other hand, immunomodulating peptides of animal origin, such as the thymic hormones and tuftsin, can rightly be considered as endogenous immunomodulators and this may prove to be true also for the peptides obtained from colostrum or from milk, whereas the immunosuppressive activities of peptidic fragments from fibrinogen or from β_2-microglobulin may have pathological relevance. Finally, it is increasingly clear that some neuropeptides must play a role as transmitters of signals from the central nervous system to the immune system.

Some muramyldipeptide derivatives, like murabutide, and lipopeptides, like pimelautide, are presently undergoing phase I clinical trials; on the other hand, thymic hormones, tuftsin and bestatin are already rather widely used in immunotherapy protocols aiming at treating patients with immunodeficiencies, cancer and rheumatoid arthritis. It is of course too early to venture any prediction about the place that these agents will occupy in tomorrow's therapeutic strategies and to guess the field in which they will be most useful. Ciclosporine, however, already represents significant progress in pharmacological immunosuppression and is being found useful in an increasing number of pathological situations. If neuropeptides are indeed signal transmitters between the central nervous and the immune systems, one may anticipate a pharmacological exploitation of this situation if and when the general pharmacological activities of these peptides become better known.

With the exception of some thymic hormones, we have restricted this review to peptides of small mol. wt, thus intentionally precluding any discussion of the various 'soluble mediators' of immunity which have been discovered more or less recently (interleukins IL-1, IL-2, IL-3; γ-interferon, B cell growth factors, etc.), some of which may well be useful in the future as immunomodulating drugs. The fact that, as we have shown, much smaller peptides are capable of exerting powerful effects on the immune system may encourage investigators to study the biological activities of peptidic fragments obtained through cleavage of the larger molecules or through synthesis of relevant sequences. Chemical synthesis of non-peptidic structural analogs of some of the main immunomodulating peptides may also be envisaged.

A good number of peptides, generally of high mol. wt, are already on the drug market (insulin, glucagon, factor VIII, oxytocin, etc.); they sometimes create problems of administration and delivery associated with their size: such problems should not be encountered with the small immunomodulating peptides mentioned in this review.

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Suitability of urethane anesthesia for physiopharmacological investigations. Part 3: Other systems and conclusions

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Summary. The suitability of urethane anesthesia for physiopharmacological experiments in various systems is briefly reviewed. Urethane anesthesia appears to be suitable for various types of studies on respiratory function and on reflex activation of motility of the urinary bladder and some sections of the intestinal tract. However, urethane produces a variety of potentially disturbing side-effects at endocrine and renal level.
Key words. Urethane; anesthesia; physiology; pharmacology; in vivo experiments; reflexes.

This paper completes a series[48,49] dealing with the suitability of urethane anesthesia for physiopharmacological investigations.

Effect of urethane on respiratory function

Douglas et al.[16] reported a marked decrease (about 40%) of respiration rate and minute volume in guinea pigs receiving i.p. urethane (1.5 g/kg) but Advenier et al.[1], using a lower dose (1.0 g/kg i.v.), found only a small depression of respiratory function.

Sapru and Krieger[67] reported that i.v. urethane (0.75 g/kg) does not affect respiratory rate and tidal volume in decerebrated rats. Urethane (2%) has no effect on ciliary beat frequency while a higher concentration (4%) produced cilioinhibition[39].

We have observed that high concentrations of urethane (70–100 mM) are required to induce a significant depression of field stimulation-induced contractions of the rat isolated diaphragm[66]. Taken together these observations suggest that urethane, at anesthetic doses, has minimal or no depressant effect on resting respiratory function. For this reason urethane-anesthetized animals do not usually require assisted ventilation except during intrathoracic surgery or following administration of substances which depress or impair respiration. Insertion of a tracheal tube is useful to facilitate ventilation, when substances which increase saliva production (eserine, substance P etc.) are administered.

Urethane-anesthetized guinea pigs exhibit an increased pulmonary airway resistance (PAR)[1]. Since urethane has a direct depressant action on the resting tone of the isolated guinea pig trachea[50] it is conceivable that increase in PAR is mediated through a modification of the neural input to the tracheobronchial tree[1,50].

Florez and Borison[25] found that, in decerebrate cats, urethane reduces the slope of the line existing between tidal volume and alveolar pressure of CO_2 with only minimal changes in the apneic point. This was paralleled by a reduced response of the medullary respiratory integrator to electrical stimulation[9]. These findings indicated that urethane depresses the CO_2-tidal volume 'gain' mechanism[9].

Sapru and Krieger[67] reported that in rats receiving i.v. urethane (0.75 g/kg) the respiratory changes produced by injection of NaCN are depressed as compared to decerebrated rats.

Dixon and Brodie[15] reported that, in cats, urethane reduces the muscarine-induced bronchoconstriction. Douglas et al.[16] reported that bronchomotor responses to aerosolized histamine are reduced, as compared to conscious animals, in urethane-anesthetized guinea pigs.