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Short Review

Synthetic Immunoregulating Molecules: A Potential Bridge between Cytostatic Chemotherapy and Immunotherapy of Cancer

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Introduction

The concept of immunomodulation as an adjunct to cancer therapy is based on ample evidence that a variety of agents augment the immune reaction to unrelated antigens in experimental animals and increase their resistance to tumors. The ever-growing list of such agents includes viruses, bacteria, fungi, and their products (Ciba Foundation Symposium, 1973). Following the pioneering work of Mathé with BCG (Mathé, 1971), investigators have been very active in this field in recent years (Chedid et al., 1973; Lamensans et al., 1975; Lamoureux et al., 1976; Laucius et al., 1974; Leclerc et al., 1976; Mastrangelo et al., 1976; Old et al., 1959; Weiss et al., 1961; Zbar et al., 1970). Most, if not all of these studies, have been done with viable or killed microorganisms (Hersch et al., 1976; Israel, 1976; Pouillart et al., 1976), cell walls (Chedid et al., 1973; Gray et al., 1975; Yamamura et al., 1976a; Zbar et al., 1972), or very complex fractions such as methanol extract residue (MER) (Perloff et al., 1977; Yron et al., 1973) or interphase material (IPM) (Lamensans et al., 1975).

Some organisms such as BCG and *Corynebacterium parvum* have been shown to be dramatically effective in certain experimental models, but investigators are well aware of the fact that they contain a great variety of heterogenous antigens, some of which might cross-react with host tissues (Borsos and Rapp, 1973; Bucana and Hanna, 1974; Vandenbark et al., 1975). They are also endowed with undesirable pharmacologic side effects (Werner et al., 1977). Consequently, current attempts at isolating chemically well-defined fractions from whole bacteria have been given a high priority, with the justification that they may lead to preparations that retain important therapeutic effects but with diminished or no toxicity.

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In this paper, it is the aim of the authors to direct attention to newer compounds that have already been identified as the active molecules of the whole mycobacterial cell, and whose synthesis has recently been effected. Together with a discussion of other adjuvants, we advance the hypothesis that certain of these synthetic substances are able to regulate the immune response because of their structural analogy to molecules native to the parasitic agent or to the host.

Synthetic Immunoadjuvants with Structural Analogy Related to Microorganisms

Development of these synthetic compounds originated mainly through studies of mycobacteria, which were utilized extensively by several investigators because of their strong, multiple effects on the immune response. Although the capacity to immunize specifically against tuberculous infection is restricted to certain strains of this organism, nonspecific resistance to infections (Blanden et al., 1969; Dubos and Schaedler, 1957; Howard et al., 1959), as well as enhancement of tumor immunity (Lamensans et al., 1968; Laucius et al., 1974; Mathé, 1971; Old et al., 1959; Zbar et al., 1971), has been elicited by a large variety of mycobacteria. As was shown by Freund, these organisms are also capable of inducing delayed hypersensitivity to many antigens when administered with them in a water-in-oil emulsion, in addition to their property of markedly increasing humoral antibody levels (Freund, 1956; White, 1976). Thus, a common adjuvant unit was suggested, which was rather widely dispersed throughout the mycobacteria genus. However, the utilization of whole mycobacterial cells as adjuvants became restricted because of their multiple side effects, and because the nature of their immune modulation was unpredictable, e.g., tumor enhancement instead of rejection (Werner et al., 1977). Clinical application has also been severely limited by the requirement

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for a nonmetabolizable oil as a component of Freund's complete adjuvant (FCA) (Holt, 1967). These realizations have served as a continuing stimulus to fractionate mycobacterial cells and identify and synthesize the active, immunoregulating molecules devoid of toxicity and immunogenicity, such that they might be useful tools for clinical application.

The difficult problem of determining the chemistry of fractions with adjuvant activity from mycobacteria had been pursued for many years, but only recently have the important, relevant structures been identified. Early investigations revealed that the whole organisms could be replaced by a wax, termed Wax D, which was extracted and solubilized in lipid solvents (Raffel, 1948; White et al., 1964), or by purified cell walls (Azuma et al., 1971). More recently, an important advance has been made in the identification of water-soluble fractions (Adam et al., 1972, 1973; Hiu, 1972; Migliore-Samour and Jollès, 1972; Stewart-Tull et al., 1975). Thus, a preparation containing arabinogalactan linked to peptidoglycan was obtained after lysozyme treatment of *Mycobacterium smegmatis* by Adam et al., and found capable of substituting for whole mycobacterial cells in Freund's complete adjuvant (Adam et al., 1972, 1973; Hiu, 1972; Migliore-Samour and Jollès, 1972; Stewart-Tull et al., 1975). In contrast to whole cells or to crude cell walls, this fraction, termed Water Soluble Adjuvant (WSA), did not induce side effects (Chedid et al., 1972) and was not arthritogenic, although it could induce delayed hypersensitivity and, under some circumstances, autoimmune diseases (Lebar and Voisin, 1974; Toullet et al., 1974). In vitro experiments have shown that WSA amplified the immune response to T-independent antigens, and that this activity might be exerted at the level of the macrophage (Modolell et al., 1974). However, this adjuvant may have multiple effects in that WSA has been shown to be active in the mixed lymphocyte reaction (Bona et al., 1974) and to be capable of restoring the immune response in mice depleted of antibody-forming cells (Liacopoulos et al., 1974). In these instances, mediation may occur through the thymus-derived cell.

Further chemical breakdown of WSA revealed that the minimal cell wall subunit capable of replacing whole mycobacterial cells in Freund's complete adjuvant was a monosaccharide tripeptide (Ellouz et al., 1974). A portion of the latter (N-acetyl muramyl-L-alanyl-D-isoglutamine, MDP) was subsequently synthesized and shown to be the minimal unit with immunoregulatory properties (Adam et al., 1975; Ellouz et al., 1974; Kotani et al., 1975a; Merser et al., 1975). The formula for WSA and MDP are shown in Figure 1, which depicts their relationship to the mycobacterial cell wall skeleton and Wax D structure as stipulated by Lederer (Lederer, 1977; Lederer et al., 1975).

In contrast to previous adjuvant fractions, MDP was found to be very active when injected in an aqueous medium with antigens (Audibert et al., 1977a, 1976). Moreover, its effects were demonstrable by several routes, including oral administration (Chedid et al., 1976). Various aspects of the biological activity of MDP and its analogues have been reviewed recently (Chedid and Audibert, 1977; The Lancet, 1977) and its mechanisms of action studied in several systems (Damais et al., 1977, 1978; Juy and Chedid, 1975; Liacopoulos et al., 1974; Specter et al., 1977; Sugimoto et al., Yamamura et al., 1976b). Thus, MDP has been shown to activate macrophages in vitro, enabling them to inhibit the growth of tumor target cells (Juy and Chedid, 1975). Oppenheim and Mizel have observed that MDP induces mitogenic activity in the supernatant fluids of mouse peritoneal exudate cells and human peripheral mononuclear cells. This activity resulted when the cell population was enriched for adherent cells and is therefore like-

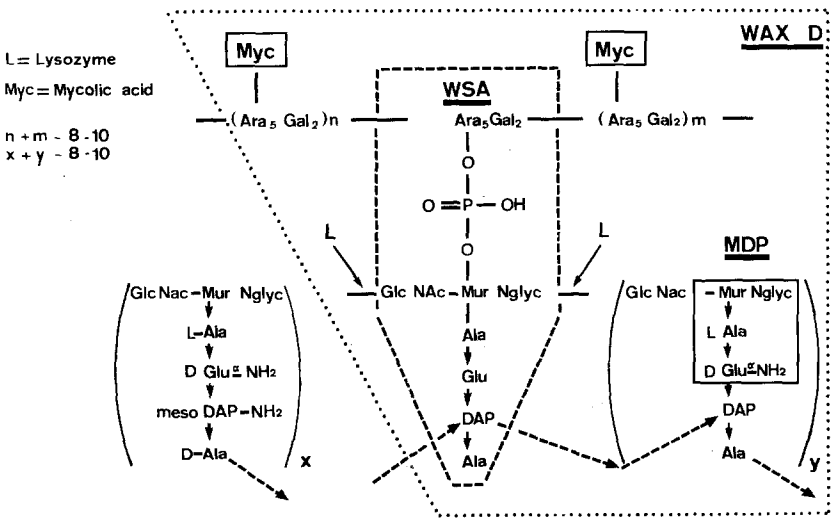

Fig. 1. Simplified scheme of the mycobacterial cell wall and its adjuvant-active derivatives wax D, water-soluble adjuvant (WSA), and N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP). The WSA unit shown here has a molecular weight of about 2000. Myc: mycolic acid; L: lysozyme; according to E. Lederer (1977)

ly to be similar to lymphocyte-activating factor 'LAF', although the adherent cells contained some lymphocytes and therefore also may be making mitogenic factor (unpublished results).

Administration of MDP with SRBC enhances the titer of circulating antibodies by acting on T-lymphocytes (Löwy et al., 1977). More recently, using trinitrophenyl-ovalbumin and trinitrophenyl-keyhole limpet hemocyanin as a carrier hapten system, it was observed that the effect was mediated by carrier-specific T-helper cells (Sugimoto et al.).

Most of the data obtained when MDP was injected into saline, utilized systems concerned with humoral antibody production. When administered into Freund's incomplete adjuvant (FIA), MDP, like WSA, was found to induce delayed hypersensitivity (Azuma et al., 1976; Ellouz et al., 1974; Kotani et al., 1975b; Merser et al., 1975; Tanaka et al., 1977b) and even autoimmune diseases (Nagai, 1976; Toullet et al., 1977). Its potency with respect to the latter was evidenced when it was shown that FIA containing MDP administered with a synthetic encephalitogenic peptide antigen was more powerful than FCA (Nagai, 1976).

Recently, Yamamura has shown that allogeneic cell-mediated cytotoxicity was enhanced by MDP in vitro but not in vivo whereas a mycoloyl-MDP derivative was active in vivo (Yamamura et al., 1976b). In these studies the synthetic molecules were administered in an aqueous medium. These results argue favorably for the potential use of such agents in cell-mediated immunity and consequently in immunotherapy of cancer.

Multiple analogues of MDP with minor modifications are now being synthesized, and correlations between biological activity and chemical structure are beginning to be made (Audibert et al., 1977b; Chedid et al., 1976; Damais et al., 1978). To illustrate, certain derivatives, such as an MDP stereoisomer where D-alanine was substituted for the L form, were found capable of inhibiting the immune response, suggesting their possible use as immunosuppressants (Adam et al., 1976; Chedid et al., 1976). It is also of importance that certain of these synthetic analogues stimulated nonspecific resistance to infection (Chedid et al., 1977) and increased phagocytosis (carbon clearance) (Tanaka et al., 1977a).

Although MDP was shown to be devoid of toxicity in normal or even adrenalectomized mice (Chedid et al., 1977), it was surprisingly found that this molecule and some of the adjuvant active analogues induced a febrile response in rabbits (Kotani et al., 1976).

It was also reported that MDP incubated with granulocytes or monocytes recovered from humans or rabbits induced the production of endogenous pyrogen in vitro. The same molecules used at high dosage levels were also capable of coagulating a limulus lysate (Dinarello et al., 1977). It must be noted, however, that two analogues that have the same degree of adjuvant activity had a slight or even no detectable pyrogenicity under the same conditions (Dinarello et al., 1977).

Synthetic Immunoadjuvants with Structural Analogies to Native Substances

A second distinctive group of synthetic immunoregulatory molecules were tested or produced initially because of their structural analogies with natural molecules of the host or the parasitic agent. For example, Munder et al. observed that, after stimulation by various adjuvants, an increased formation of lysolecithin by macrophages occurred. Consequently, exogenous lysolecithin, as well as several analogues, were tested and shown to be active as adjuvants in vivo, augmenting antibody titers to various antigens (Munder et al., 1976; Westphal et al., 1970).

A somewhat similar rationale guided the investigation a few years earlier of the effect of synthetic polynucleotides on the immune response (Johnson, 1976). Initially, nucleic acids were explored as the possible endogenous host mediator of the potent adjuvant action of the endotoxic lipopolysaccharides isolated from gram-negative bacteria (Merritt and Johnson, 1965). Thus, the cytotoxic action of the endotoxin in vivo was hypothesized to cause the release of cellular nucleic acids in the vicinity of immunocompetent cells, which were being called upon for a burst of nucleic acid synthesis as a result of concurrent antigen stimulation (Kind and Johnson, 1959). The postulate that the released nucleic acids might serve as trephocytic molecules to help meet this demand and accentuate synthesis was supported by the subsequent demonstration that stimulation of the immune response to unrelated antigens was a property of both endogenous and exogenously derived nucleic acids, as well as of the low molecular weight breakdown products isolated following treatment of nucleic acids with nucleases (Johnson and Hoekstra, 1967; Merritt and Johnson, 1965). Subsequently, Braun and his colleagues, guided both by their previous extensive studies showing oligonucleotides capable of stimulating division in bacterial cells, and by their postulate that mammalian antibody synthesis might require endogenous stimulatory molecules of a similar nature (Braun and Firshein, 1967), recognized and tested the potential of the newly developed (1965) synthetic polynucleotides. Their demonstration that polyinosinic, polycytidylic (poly I : C) and polyadenylic, polyuridylic acid (poly A : U) complexes were capable of increasing the numbers of antibody-forming cells 4–5 fold caused interest to focus rapidly on these synthetic complexes because of their defined structure and ready availability (Braun and Nakano, 1967).

Conversely, Hilleman et al. (1971), who were searching for the viral component responsible for the inducement of interferon in vivo, also explored the polynucleotides early as possible synthetic substances mimicking parasitic activity. Their studies showed the double-stranded nucleic acids and their synthetic counterpart, poly I : C, to be convincingly active in this respect.

The polynucleotide complexes have been found to be adjuvants in mice, rats, guinea pigs, and rabbits, and their activity has been established for a wide range of thymus-dependent antigens. The signal from the adjuvant is apparently received very rapidly by the host inasmuch as the complexes are destroyed within 5–10 min by the nucleases in serum (Schmidtke and Johnson, 1971). These adjuvants have proved to be very effective T-cell stimulants with respect to both T-helper (Cone and Johnson, 1971) and suppressor cells (Morris and Johnson, 1977), as well as to cytotoxic cells (Bick and Möller, 1977). They show distinctive temporal requirements in that injection of this adjuvant together with an antigen increases antibody synthesis (Schmidtke and Johnson, 1971). In contrast, injection of poly A : U 1 or 2 days prior to injection causes the formation of suppressor cells with a resulting diminution of antibody synthesis (Morris and Johnson, 1977). Thus, these synthetic molecules can be considered true regulators of the immune system. Complexing of the base pairs has been shown to be a requirement in most instances (Schmidtke and Johnson, 1971), but when added to Freund's incomplete adjuvant, poly U alone was capable of increasing delayed hypersensitivity in the guinea pig (Paterson and Drobish, 1975) and of sensitizing the rat for allergic encephalomyelitis. However, artifactually induced double-strandedness has not been ruled out in these instances (DeClercq and Merigan, 1969).

Evidence has been gained that poly A : U functions as an adjuvant by causing T-lymphocytes to hasten their secretion of T-helper factors (Bick and Johnson, 1977). Such soluble helper factors secreted under the influence of poly A : U have been shown to increase the number of antibody-forming cells in the spleen, and were active in restoring the deficient immune response of aging mice (Han and Johnson, 1976) and preventing the development of tolerance in normal mice (Fessia et al., 1977). In addition, poly A : U has been shown to increase markedly the maturation of the immune system in the neonatal mouse (Han and Johnson, 1976).

Little, if any, toxicity has been displayed by poly A : U. On the other hand, poly I : C has proven to be toxic to intestinal tissue, pyrogenic, and lethal for mice, and induce hemorrhagic necrosis in kidneys (Han et al., 1973; Philips et al., 1971).

Recently, it has been shown that human peripheral blood leukocytes (PBL) double the synthesis of immunoglobulin when stimulated by poly A : U. PBL from patients with acquired agammaglobulinemia similarly showed elevated Ig synthesis under the influence of polynucleotides. However, such cells from patients with congenital agammaglobulinemia could not be elevated in their synthetic capacity by poly A : U (Lederman et al., 1977).

Both polynucleotide complexes have been shown to be effective stimulants of the immune system against certain tumors (Johnson, 1976). However, the lack of toxicity of the poly A : U complex may be an important determinant of priority in this respect. Field trials are under way in human beings to measure the effectiveness of poly A : U on the immune response of tumor-bearing patients as well as of patients who have undergone tumor removal by surgery (Lacour et al., 1974).

Synthetic Drugs as Immunoadjuvants

Within the past decade, other synthetic compounds have been shown to stimulate immunity. However, in contrast to MDP, its analogues, and the polynucleotides, these molecules were tested initially because of their various pharmacologic activities rather than being derived from active microorganisms. For example, Mayer and Fink (Mayer and Fink, 1970) have shown that bis-(diethylamino-ethoxy)-fluoren-9-one (tilorone) induced the immunoregulatory molecule, endogenous serum interferon, even on oral administration. Further experimentation has shown that tilorone is capable of enhancing antibody responses to sheep red blood cells (Diamantstein, 1973) and of suppressing delayed hypersensitivities (Megel et al., 1974).

In addition, another synthetic compound of low molecular weight, tetramisole, was shown by Renoux and Renoux in 1971 to augment the protective effect of a Brucella vaccine in mice (Renoux and Renoux, 1971). This compound had been available several years previously as an antihelminthic drug. Levamisole hydrochloride is the levarotory enantiomer of tetramisole and appears to have similar effects on the immune system (Renoux and Renoux, 1971). This observation prompted intensive investigative efforts, both by immunologists and clinicians, resulting in more than 400 papers being published in this short period of time (Symoens, 1976; Symoens and Rosenthal, 1977).

The cumulative evidence from studies on isolated cells, experimental animals, healthy volunteers, and patients suggests that levamisole restores to normal the functions of phagocytosis and T-lymphocytes in immunodeficient hosts. Although this compound has been studied extensively by various investigators for its effect in oncologic diseases, there still exist many conflicting

data in the literature. A very complete review of this subject has been published by Symoens and Rosenthal (1977). In addition, Oettgen et al. have discussed levamisole thoroughly in the recent Symposium on Immunotherapy of Malignant Disease (Oettgen et al., 1976).

The trend toward synthetic adjuvants is emphasized further by the recent studies of 4-imino-1.3-diazabicyclo-(3.1.O)-hexan-2-on, Boehringer Mannheim (BM06002), by Micksche (Micksche et al., 1977). This adjuvant has been reported to be active for both humoral and cell-mediated immune reactions in animal models, and to stimulate immune reactions in man.

Discussion

The nature of adjuvant material has long been considered to be foreign, aggressive, or insulting to the host. Consequently, it was expected that only complex, insoluble, undegradable materials were capable of enhancing the immune response by nonspecific mechanisms. Since Freund's complete adjuvant consistently has been documented as the most potent agent in this respect, it was postulated that whole mycobacterial cells or cell walls were required for its manifestation. The discovery that Wax D, a well-defined material, could substitute for the whole cell did not alter this view considerably, inasmuch as Wax D contains mycolic acid, which represents a unique mycobacterial constituent and renders this material difficult to be disposed by the host. Even the discovery of the activity of WSA and the native monosaccharide tripeptide subunit was consistent with the requirement for a foreign structure, since diaminopimelic acid (DAP), D-isoglutamine, and N-acetylmuramic acid do not exist in eukaryotes. However, with the knowledge that removal of DAP still did not abolish the adjuvant activity, it became more apparent that extremely small and simple molecules with minimal differences from the host's own structures were sufficient for adjuvanticity. The fact that regulation of the immune response now can be achieved with such small peptides raises the question as to whether these molecules function by virtue of possessing structures similar to those existing in the host. One might boldly pose the question "Does MDP and its analogues represent man's synthetic version of a natural lymphokine?" (Barksdale and Kim, 1977).

It is important to recognize in this respect that lymphokine structures are unknown and that only a few immunoregulatory molecules of the host other than immunoglobulin, thymic serum factors, and thymosin alpha 1 have been sequenced. The latter two were recently also synthesized. As a matter of fact, the synthesis of analogues of bacterial products with immunologic activity may represent an easier approach to the acquisition of knowledge of natural structures. It is of interest that D-isoglutamine, which is essential for the activity of MDP, is not hydrolyzable by enzymes of mammalian hosts and thus cannot be degraded. Substitution of the L-isomer at this point results in an inactive adjuvant. One might then speculate that the nondegradable synthetic product may be a distinct asset as compared with the natural mediators, which probably have short biological half-lives.

We attach conceptual importance to the omission of the word 'immunoadjuvant' from our title. It expresses our feeling that a new era in studies of adjuvants has dawned in which these substances are recognized as having a much broader regulatory activity in inducing suppression as well as in enhancing the immune response (Schwab, 1975). It is of interest in this respect that in his recent review, Schwab stated that "Almost every material having adjuvant activity can also be demonstrated to suppress the immune response with appropriate manipulation of timing, dose and selection of antigen. The greater significance of these agents may be as immunosuppressants." Several adjuvants have now been demonstrated to be active immunosuppressive agents in animals at microgram levels as compared with the milligram doses required with cancer therapeutic drugs. Whereas all known bacterial adjuvants such as BCG and *C. parvum,* etc., have considerable antigenic diversity, the functionally pure fractions now available and described herein should provide distinctive probes for definitive studies of immunoregulatory mechanisms.

Experimental analysis of the structural control of immunogenicity has shown that the humoral response to DNP-oligolysines was dependent on the *kind* of mycobacteria used in FCA (Stupp et al., 1971). Under certain well-defined conditions, the magnitude of the antibody response was independent of the PLL gene status of the guinea pigs, but was related to the type of mycobacteria used ($H_{37}Rv$ instead of *M. butyricum*). Similarly, Nagai (1976), using an analogue shorter than the nonapeptide encephalitogen antigen, has presented evidence that MDP selectively induced this autoimmune disease, whereas FCA with whole mycobacteria could not. Therefore, such well-defined synthetic adjuvants should also permit a more precise appraisal of the genetics of the immune response. Up to the present time, these studies have been undertaken necessarily with very complex adjuvant materials.

The addition to tumor therapy of immunoregulating substances such as those reviewed herein may be advocated for multiple reasons. For example:

(a) enhancement of specific immunization by tumor antigens,

(b) enhancement of nonspecific immunity active against tumors,

(c) use of the immunosuppressive capacity of certain analogues to reduce tumor-blocking or tumor-enhancing antibodies, and

(d) restoration of immunocompetence following a deficiency induced by the cancer itself or by cancer chemotherapy.

Man has the habit of interpreting Nature in ways more difficult than she intended. For a long time, there have been conceptual differences between chemotherapy and immunotherapy, sometimes to the point of the two being in opposition. Yet it must be recalled that cancer chemotherapy has rendered available for decades several *synthetic cytostatic compounds which were also immunosuppressive* as recently reviewed in detail (Schwab, 1975). The gap existing between chemical and immunologic approaches to tumor therapy also appears to be bridged by the availability of the synthetic compounds emphasized herein that represent analogues of natural structures existing in the host or parasite and that are capable of regulating the immune response. The rationale for use of polynucleotides as adjuvants has been their relationship to natural products existing in the host or in viruses. Similarly, it has been suggested by Barksdale (Barksdale and Kim, 1977) that MDP could "do its job because it mimics a regulator molecule of the immune system." Whether such is the case remains to be demonstrated; however, the recognition of the emergence of low molecular weight monosaccharide peptides, representing an extraordinary small unit capable of replacing mycobacteria in Freund's adjuvant, should help to establish an important bridging relationship between chemical and immunologic tumor therapy.

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