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## Hydrosoluble Immunostimulants of Bacterial and Synthetic Origins

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*Summary.* The role of whole *Mycobacteria*, mycobacterial cell walls and waxes D as immunostimulants was well established many years ago. More recently three different research groups have shown that *hydrosoluble* components from mycobacterial and other bacterial origins were as active as waxes D or cell walls and were free of many side-effects. Studies concerning the relationship between structure and activity were achieved which led to the description of a small biologically active fragment and to a first series of synthetic compounds.

### 1. *Introduction*

In the present review we want to bring up to date and to expand the data which we published in 1973 in the book 'Chemical and Biological basis of Adjuvants'[2] concerning the hydrosoluble immunostimulants of bacterial origin, and to discuss also some related synthetic compounds. Indeed many new facts have come to light since then and more and more research has been devoted to the possible use of immunostimulants in the treatment of bacterial and viral diseases and of some forms of cancer.

The role of *Mycobacteria* as adjuvants was found indirectly from the observation of LEWIS and LOOMIS[3,4] that a higher titer of haemolysin was produced in tuberculous than in normal guinea-pigs. Later FREUND et al.[5] observed that the addition of paraffin oil to killed tubercle bacilli stimulated antibody formation against protein antigens; several stains of *Mycobacteria* were found adjuvant-active by FREUND[6].

The next step in this study was the characterization of the active principle of whole mycobacterial cells. A marked adjuvant activity in both the production of circulating antibody and the development of a delayed hypersensitivity to ovalbumin was found by WHITE, BERNSTOCK, JOHNS and LEDERER[7] and WHITE, JOLLÈS,

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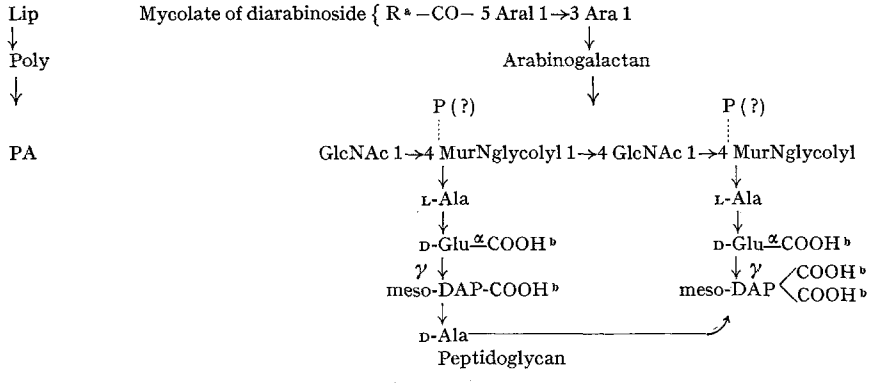
Table I. The structure of wax D and of an adjuvant-active hydrosoluble peptidoglycan (tetrasaccharide-heptapeptide, TH) from *M. tuberculosis* var. *hominis*




Lip→Poly→PA: wax D (peptidoglycolipid)[2]. Poly→PA: hydrosoluble adjuvant of higher molecular weight (> 10,000)[16]. PA: peptidoglycan or tetrasaccharide-heptapeptide (TH)[30,31]. [a] Mycolic acid. [b] 2–3 Carboxylic groups are amidated.

SAMOUR and LEDERER[8] by using a peptidoglycolipid fraction called wax D from human strains of *M. tuberculosis*. The lack of activity of other types of lipid fractions from *M. tuberculosis* var. *hominis* attested to the specific activity of wax D or its components[2,9]. An active wax D (Table I) contains a lipidic part mainly constituted by mycolic acids (Myc) esterified to a polysaccharide (Poly; mainly an arabinogalactan) and a 'nitrogen-containing' moiety (a peptide linked to aminosugars, called PA)[2,8,10]. The occurrence of biologically inactive wax D preparations of some bovine strains of *M. tuberculosis* (BCG, Marmorek) or of saprophytic strains of *Mycobacteria* such as *M. smegmatis* has been observed[9,11]: the principal chemical difference noted between an active and an inactive wax D is that the former has a peptide and amino sugar moiety and the latter has not[2,8]. An important problem was to elucidate the chemical structure of this 'nitrogen-containing' moiety: it was established by MIGLIORE and JOLLÈS[12,13] and turned out to be closely related to the peptidoglycan which constitutes the backbone of mycobacterial as well as other cell walls (molar proportions of N-acetylglucosamine: N-acetyl (or N-glycolyl) muramic acid:Ala:Glu:DAP, 2:2:3:2:2). Thus it was not surprizing to find that pure cell wall preparations were also able to replace whole mycobacterial cells in Freund's adjuvant[14,15].

A new chapter in this whole research field was opened when three different groups showed independently in 1972[15–17] that *hydrosoluble* products were as active as wax D or cell walls and were free at least in part of side-effects such as production of polyarthritis observed with whole mycobacterial cells. Studies concerning the relationship between structure and activity were achieved which led to the description of a small biologically active fragment (glycopeptide) and to a first series of synthetic compounds.

In connection with the various hydrosoluble compounds which will be mentioned in the following sections, several biological properties will be discussed when possible, more particularly adjuvant activity[18,19] (stimulation of the production of antibodies against the antigen used; induction of delayed hypersensitivity to the antigen used); antiviral activity; mitogenic activity.

## 2. *Hydrosoluble compounds with a molecular weight higher than 10,000*

2.1. *Substances from Mycobacteria.* Three French research groups[15–17] published independently in 1972 three different preparation procedures of high molecular weight hydrosoluble adjuvants.

2.1.1. *Preparation techniques.* The chemical structure studies achieved by MIGLIORE and JOLLÈS[12,13] strenghtened the view that the adjuvant-active wax D could be some degradation product of mycobacterial cell walls; these authors concluded that this enzymic degradation (auto-digestion) might result naturally in a hydrosoluble moiety. They described[16] a simple and mild extraction technique of such a hydrosoluble adjuvant-active fraction starting directly from delipidated mycobacterial *cells* such as *M. tuberculosis* var. *hominis*, strain Peurois. Further purification was achieved by chromatography on DEAE-cellulose and filtration on Biogel P 10. On the basis of 3 residues of Ala/mole, a molecular weight of 14,800 was calculated. The amino acid and amino sugar contents were 6.2 and 7%, respectively; the neutral reducing sugars (Ara, Gal, Man) constituted the remaining part, as the lipid content was lower than 0.5%. These analytical data were in favour of a close relationship between this hydrosoluble compound (Poly-PA, see Table I) and the hydrosoluble moiety obtained by saponification of wax D.

ADAM et al.[15] isolated a macromolecular, water-soluble fraction from trypsin and chymotrypsin treated delipidated *cells walls* of *M. smegmatis* which were digested overnight with lysozyme. The hydrosoluble products thus obtained were filtered on Sephadex G-50. The material of the first peak (WSA) behaved in the ultracentrifuge as a slightly polydisperse macromolecule with an approximate molecular weight of 20,000. All the constituents of WSA were typical bacterial cell wall constituents; thus WSA was considered to be an 'oligomer' of the cell wall.

HIU[17] prepared similar hydrosoluble products from hydrogenolysis products of BCG.

2.1.2. *Biological activities.* The 'Poly-PA' prepared by MIGLIORE and JOLLÈS[16] possessed an adjuvant activity when added to Freund's incomplete adjuvant (FIA) with an antigen (ovalbumin). It no longer elicited the arthritis-inducing effect observed with wax D[16]. Several other biological properties of this compound were established by WERNER et al.[20]. Poly-PA increased the production of anti-influenza virus antibodies in the rabbit; injected in the mouse, it increased the number of antibody-producing cells in the spleen

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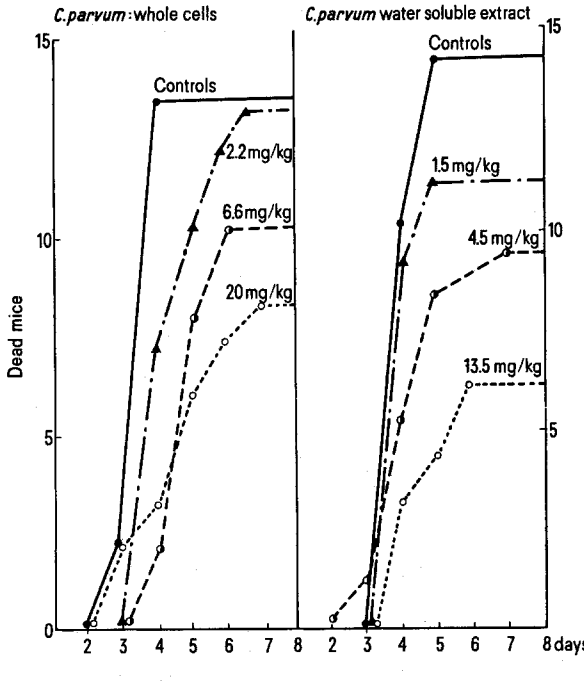
Table II. Summary of activities in various immunological tests of hydrosoluble substances from *C. parvum*[25].

| Preparation | Mouse spleen plaque-forming cells | Carbon clearance in the mouse | Hypersensitivity in the guinea-pig to | | Anti-influenza virus HAI Antibodies (rabbit) |
|---|---|---|---|---|---|
| | | | Ovalbumin | ABA-tyrosine | |
| Crude watersoluble extract | 0.46[a] | 3.23[c] | 25.6[d] | 89[e] | 10[f] |
| Purified watersoluble substance | 0.28 | 3.03 | 4.8 | n.d. | 4 |
| *C. parvum* whole cells | 0.74 (i. p.) | 4.2 | 32.4 | n.d. | n.d. |
| FCA (Freund's complete adjuvant) ($H_{37}Ra$) | 0.56[b] (s.c.) | n.d. | 38.5 | 91 | 4 |

[a] Ratio: Average of number of plaques in spleens of control mice over average number of plaques in spleens of treated mice (10 mice in control series, 5 mice in treated series).
[b] FCA contained *M. phlei* instead of the $H_{37}Ra$ strain of *M. tuberculosis*.
[c] Ratio: Clearance rate ($K$-value) in treated mice over clearance rate in control mice (2 animals used in each series for each time interval).
[d] Ratio: Square diameter ($mm^2$) of erythematous reaction (48 h after skin test) in guinea-pigs immunized with ovalbumin + FIA + substance under study over surface of erythema in animals immunized with ovalbumin + FIA alone (3 guinea-pigs per series).
[e] Square diameters of erythematous reaction (24 h after skin test, average of 3 guinea-pigs) in animals immunized with ABA — tyrosine + FIA + substance under study. Control (ABA — tyrosine + FIA) animals give no reaction in this test.
[f] Ratio: Average serum HAI titer in rabbits immunized with vaccine + FIA + substance under study over average titer in rabbits immunized with vaccine + FIA alone (3 rabbits per series).

and enhanced the graft versus host reaction; an increase of DNA synthesis when present in in vitro cultures of leucocytes from healthy human subjects was also noted.

WSA[15] also induced hypersensitivity to ovalbumin in the presence of FIA; furthermore it increased the amount of precipitating antibodies and induced the biosynthesis of $\gamma_2$ type precipitating antibodies and was effective in increasing the immune response to viruses[21]. WSA had a stronger adjuvant activity than equal amounts of whole bacteria, cell walls or wax D; the absence of undesirable effects such as allergic polyarthritis in rats or induction of hyper-reactivity to endotoxin was verified. WSA proved also to be an adjuvant in vitro[22]. It increased the immune response of mouse lymphoid cells against sheep red blood cells, DNP-dextran and DNP-edestin and seemed to act in vitro primarily on macrophages.




Activity of *Corynebacterium parvum* whole cells and of crude water-soluble extracted material on infection of CD-1 mice with encephalomyocarditis virus. One i.v. treatment 2 h before virus inoculation. Curves represent cumulative mortality.

The fully water-soluble lipid-free fraction described by HIU[17] exhibited also a marked adjuvant-effect on the production of immune antibody to sheep red blood cells. MEYER et al.[23] have shown that both WSA and HIU's extract were active in experimental allergic encephalomyelitis production.

A water-soluble mycobacterial glycopeptide was obtained in large quantities from the culture supernatant fluid of *M. tuberculosis* strain DT. This glycopeptide was strongly adjuvant-active when injected in a water-in-oil emulsion containing ovalbumin into guinea-pigs[24].

*2.2. Substances from various bacterial origins.* Active water-soluble substances have been obtained by MIGLIORE-SAMOUR et al.[25] from delipidated cells of *Corynebacterium parvum* (strain Prévot). Immunostimulating and adjuvant activities were demonstrated by various procedures, including in vivo techniques involving both cell-mediated and humoral (Table II) immune reactions. Partial, but dose-dependent, protection against encephalomyocarditis virus was observed (Figure) as well as a reduction of the Moloney

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sarcoma virus induced splenomegaly in BALB/c mice. The hydrosoluble extract exerted an immunopotentiating effect as marked as, and in some instances higher than, that of a suspension of whole cells.

Adjuvant acitvity was also observed with hydrosoluble extracts from *Nocardia* strains[26]. The mitogenic activity of hydrosoluble fractions from lysozyme extracts of delipidated cells from three strains of *Nocardiae*[27] has been established by BONA et al.[28] who have shown that they stimulated B lymphocytes.

Table III. Some biological properties of the acetylated tetrasaccharide-heptapeptide (TH; peptidoglycan, see Table I)[30]
A) Hypersensitivity to ovalbumin in the guinea-pig

| Immunization with | Activity index[a] on cutaneous reaction (48 h) to 10 µg ovalbumin |
|---|---|
| 1 mg ovalbumin + FIA | 1 |
| 1 mg ovalbumin + FIA + 0.5 mg killed H37Ra bacilli | 38.5 |
| 1 mg ovalbumin + FIA + 0.5 mg TH | 52.3 |

B) Adjuvant effect on production of hemagglutination-inhibition (HAI) antibodies against influenza virus (inactivated B/Mass. 3/66) in the rabbit

| Antigen emulsified in | $Log_2$ HAI antibodies: (individual values) |
|---|---|
| Antigen alone | 7, 9 |
| FIA | 10, 10, 11 |
| FIA + 1 mg TH | 12, 12, 12 |
| FIA + 0.1 mg TH | 11, 13 |
| FIA + 1 mg killed H37Ra bacilli | 11, 12, 13 |

C) Effect on antibody-producing cells in mouse spleen

| Experiment 1 Compound | Daily dose[b] (mg/kg i.v.) | Activity index[c] |
|---|---|---|
| TH | 25 | 0.93 |
| | 50 | 0.75 |
| BCG | 25 | 0.34 |
| | 50 | 0.27 |
| **Experiment 2 Compound** | **Daily dose[b] (mg/kg i.v.)** | **Activity index[c]** |
| TH | 25 | 0.39 |
| BCG | 25 | 0.37 |

[a] Activity index: ratio of surface of erythematous reaction in animals immunized with ovalbumin + adjuvant to corresponding reaction in controls. [b] Treatment on: days 0, +1 and +2 (experiment 1); day 0 (experiment 2). [c] Ratio: number of plaques in spleens of untreated mice to number of plaques in spleens of treated mice on day +3 (experiment 1); day +2 (experiment 2).

All the immunoadjuvants hitherto reported to be active in induction of delayed hypersensitivity and in stimulation of increased circulating antibody levels seemed to contain common compounds, consisting of the characteristic amino sugars and amino acids of bacterial cell walls. It seemed likely that bacterial cell walls in general, from Gram-negative as well as from Gram-positive bacteria, and their hydrosoluble split products might have definite adjuvant activities. Using various cell wall lytic enzymes, KOTANI et al.[29] solubilized from various Gram-positive bacteria (*Staphylococcus aureus; Streptococcus pyogenes; Streptococcus salivarius; Streptococcus faecalis: Streptococcus mutans; Lactobacillus plantarum; Bacillus megatherium; Corynebacterium diphtheriae; Actinomyces viscosus*) immunoadjuvant principles with full retention in presence of mineral oil of the adjuvant activities from the walls. Evidence was obtained that the nonpeptidoglycan portion of the cell walls was not essential for manifestation of immunoadjuvanticity.

### 3. *Hydrosoluble compounds of low molecular weight and natural peptides with known structure*

3.1. *Active compounds with still a neutral non-amino sugar content.* JOLLÈS et al.[30,31] prepared adjuvant-active mycobacterial fractions of low molecular weight by two procedures: one in which the sugars remained unchanged during the preparation, the above-mentioned Poly-PA being submitted to further purifications by conventional biochemical methods, and one where the delipidated cells were directly acetylated. The compounds which were soluble in alcohol and water were further purified by filtration on Biogel P 10. The 'native' as well as the acetylated compounds, where all the sugars were modified, exerted a strong adjuvant effect when injected with mineral oil; their molecular weights ranged between 3000 and 7000. These substances contained principally the peptidoglycan and small amounts of reducing non-amino sugars. Such a compound induced experimental allergic encephalomyelitis in guinea-pigs using either bovine myelin basic protein or the encephalitogenic tryptophan peptide as antigens[32,33]. Using the acetylated adjuvant,

[26] A. ADAM, R. CIORBARU, J.-F. PETIT, E. LEDERER, L. CHEDID, A. LAMENSANS, F. PARANT, M. PARANT, J. P. ROSSELET qnd F. M. BERGER, Infect. Immun. *7*, 855 (1973).
[27] R. CIORBARU, A. ADAM, J. F. PETIT, E. LEDERER, C. BONA and L. CHEDID, Infect. Immun. *11*, 257 (1975).
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ZACHOWSKI et al.[34] were able to show that it acted only on the inside-out membrane vesicles and induced a biphasic modulation of 5'-nucleotidase and of the ($Na^+ + K^+$) stimulated $Mg^{++}$ ATPase: this result was in favour of an indirect effect of adjuvants on the enzymatic activities of plasma membranes.

In the process of purification of WSA by filtration on Sephadex G-50, ADAM et al.[15] obtained a fraction containing smaller products with only a low content of neutral sugars; it was also adjuvant active.

3.2. *Active peptidoglycans.* By their acetylation procedure, MIGLIORE and JOLLÈS[31] were able to prepare a small peptidoglycan, namely a tetrasaccharide-heptapeptide (TH; see Table I) from *M. tuberculosis* var. *hominis* (strain Peurois). It was shown to exert a potentiating effect on both B and T cells functions (Table III). First, used as an adjuvant, it increased the production of circulating antibodies in the rabbit and enhanced the appearance of delayed-type hypersensitivity in the guinea-pig: in such experimental systems, and in the presence of mineral oil, it exhibited an activity which was equal to or higher than that of Freund's complete adjuvant, while being devoid of the arthritis-inducing effect of the latter in the rat. Furthermore, injected i.v. ,the tetrasaccharide-heptapeptide had an immunostimulating activity which, in some respects, was comparable with that of live BCG: increase in the number of antibody-producing cells in mouse spleen and enhancement of the graft-versus-host reaction[30].

ADAM et al.[35,36] were able to prove that peptidoglycan fragments completely free of neutral sugars were at least as active as WSA, wax D or cell walls. They were the first to report that monomeric peptidoglycans (Table IV) prepared from *M. smegmatis* or *E. coli* cell walls could replace whole mycobacterial cells in Freund's adjuvant as concerns stimulation of antibody production toward ovalbumin and induction of delayed hypersensitivity toward this same antigen. The disaccharide-tetrapeptide from *M. smegmatis* was prepared by submitting the lower molecular weight compounds obtained by gel filtration on Sephadex G-75 of WSA to the following purification steps: chromatography on cellulose (DE 32), digestion with *Myxobacter* $AL_1$ amidase, filtration on Sephadex G-25 and preparative high voltage electrophoresis. The peptidoglycan from *M. smegmatis* contained a N-glycolylmuramic acid residue and D-Glu and meso-DAP were amidated; in the analogous fraction prepared from *E. coli*, muramic acid was N-acetylated and D-Glu and meso-DAP were not amidated; both compounds had the same activity.

[34] A. ZACHOWSKI, D. MIGLIORE-SAMOUR, A. PARAF and P. JOLLÈS, FEBS Lett. *52*, 57 (1975).
[35] A. ADAM, C. AMAR, C. CIORBARU, E. LEDERER, J. F. PETIT and E. VILKAS, C. r. Acad. Aci., Paris *278D*, 799 (1974).
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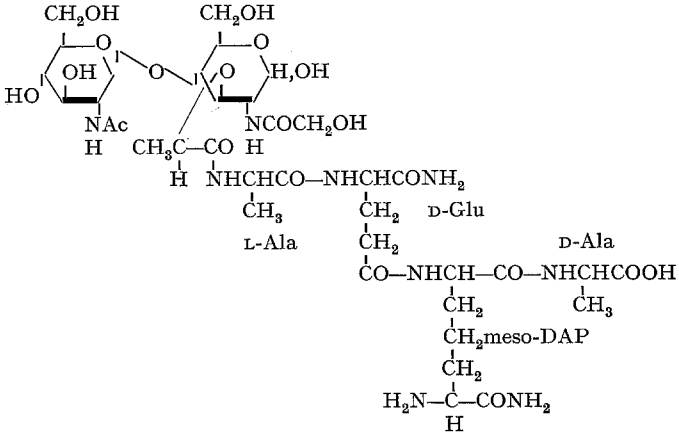
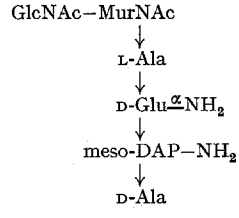
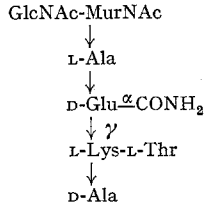
Table IV. Chemical structure of adjuvant-active hydrosoluble peptidoglycan monomers

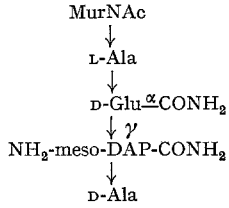


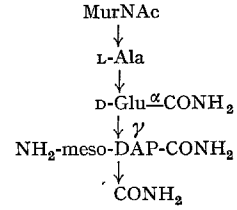




Monomeric peptidoglycan[36] from *M. tuberculosis* (left) and *E. Coli* (right)




Monomeric peptidoglycan from *M. roseus*[40]




Chemical structures of peptidoglycan subunits liberated from *L. plantarum* cell walls by various peptidoglycan-degrading enzymes[42]

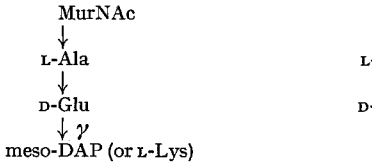
Similar results were obtained by NAUCIEL et al.[37,38] who worked with peptidoglycans obtained from *Moraxella glucidolytica, Neisseria perflava* and *E. coli* RE 600[39]. All the peptidoglycans so far studied contained meso-DAP. But it is well known that the peptide moiety of cell walls contains either meso-DAP or lysine. ELLOUZ et al.[40] evaluated the activity of lysine-containing peptidoglycans which were prepared from *Micrococcus roseus* (Table IV) and *Staphylococcus epidermidis:* both were adjuvant active. FLECK et al.[41] obtained similar results when working with the following Gram-positive bacteria: *Streptococcus faecalis, Lactobacillus casei* and again *St. epidermidis*. KOTANI et al.[42] devoted a detailed structural and biological study to the peptidoglycan monomers obtained from *Staphylococcus aureus* (DAP containing peptide) and *Lactobacillus plantarum* (Lys containing peptide) cells (Table IV) by digestions with peptidoglycan degrading enzymes: these compounds were shown to be unique chemical entities with definite adjuvant activity both in stimulating antibody production and in induction of delayed-type hypersensitivity to ovalbumin when administered to guinea-pig as water-in-oil emulsions.

3.3. *Minimal structural requirements for adjuvant activity*. A further simplification of the structure of the disaccharide-tetrapeptide (peptidoglycan monomer) was possible without losing adjuvant activity. ELLOUZ et al.[40] first showed that the C-terminal D-Ala was not necessary for the biological activity and that N-acetylglucosamine could also be removed without loss of activity (Table V). The N-acetylmuramyl-tripeptides (with a terminal DAP or Lys residue) were able to enhance the immune response when added to Freund's incomplete adjuvant (Table VI). Studies with synthetic compounds (see below) allowed, however, to demonstrate that N-acetylmuramyl-L-alanyl-D-isoglutamine was the smallest adjuvant-active substance (Tables V and VI)[40]. This result seems to be inconsistent with the observation of FLECK et al.[41] who claimed that the tetrapeptide L-alanyl-D-isoglutaminyl-L-lysyl (or meso-diaminopimelyl)-D-alanine was the smallest structure required for induction of delayed hypersensitivity. This peptide was found to be inactive by ELLOUZ et al.[40]. The major difference between these studies was that in the data of ELLOUZ et al.[40], ovalbumin was used as antigen, while azobenzene-arsonate-N-acetyl-L-tyrosine was used by FLECK et al.[41]. It should also be noted that the peptide monomer used by these latter authors was a natural product obtained, using *Bacillus licheniformis* amidase, from a disaccharide-peptide monomer of the *E. coli* peptidoglycan; the tetrapeptide employed by ELLOUZ et al.[40] was also a natural product obtained, however, by treatment of a disaccharide-tetrapeptide from *E. coli* with *Myxobacter* amidase. The results of ELLOUZ et al.[40] were confirmed by KOTANI et al.[43]. It was thus possible to suggest that a glycopeptide structure seemed to be essential for adjuvant activity. Syntheses were later undertaken in order to verify this statement.

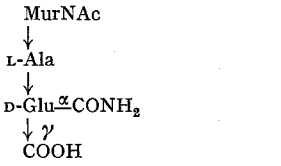
## 4. *Synthetic adjuvants. Relationship between structure and adjuvant activity*

The first biological experiment with an entirely synthetic compound was described by ELLOUZ et al.[40]. The substance, N-acetylmuramyl-L-alanyl-D-isoglutamine (Table V) synthetized by MERSER and SINAY[44,45], was at least as adjuvant-active as the monomeric unit of the peptidoglycan, while the disaccharide N-acetylglucosaminyl $\beta$ 1→4 N-acetylmuramic acid or the synthetic disaccharide-L-alanine[46] showed no activity (Table VI).

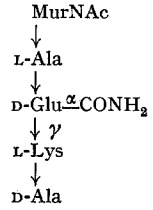
Table V. Minimal structural requirements for adjuvant activity: 3 examples of natural or synthetic active compounds




N-acetylmuramyltripeptide[40] (active)




N-acetylmuramyl-L-alanyl-D-isoglutamine[44,45] (active)




N-acetylmuramyltetrapeptide active following FLECK et al.[41], inactive following ELLOUZ et al.[40] and KOTANI et al.[47]

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Table VI. Adjuvant activities of natural or synthetic peptides or glycopeptides: some examples

| Test material | Dose (μg/animal) | Skin response (48 h) [a] Kotani et al.[41] | Skin response (48 h) [a] Ellouz et al.[40] | Antibody level (ratio) [b] Kotani et al.[47] | Antibody level (ratio) [b] Ellouz et al.[40] |
|---|---|---|---|---|---|
| GlcNAc-MurNAc | 50 | | | | 1.74 |
| L-Ala-D-isoGln-meso-DAP | 25 | | | | 1.4 |
| L-Ala-D-isoGln-L-Lys-D-Ala | 200 | 5.4 | | 1.5 | |
| GlcNAc-MurNAc-L-Ala-D-isoGln-meso-DAP | 25 | | | | 6.3 |
| MurNAc-L-Ala-D-isoGln-L-Lys-D-Ala | 12.5 | 4.4 | | 0.9 | |
| | 25 | 12.4 | | 1.5 | |
| | 50 | 12.1 | | 2.3 | |
| | 100 | 11.8 | | 2.0 | |
| | 200 | 11.7 | | 2.1 | |
| MurNAc-L-Ala-D-isoGln-L-Lys | 2 | | 11 | | 1.9 |
| | 10 | | 12 | | 4.0 |
| | 25 | 13.3 | | 3.7 | |
| | 100 | 11.7 | | 2.9 | |
| MurNAc-L-Ala-D-isoGln-meso-DAP | 5 | | 12 | | 5.4 |
| | 25 | | 14 | | 6.0 |
| MurNAc-L-Ala-D-isoGln | 5 | | 11 | | 5.1 |
| | 12.5 | 10.8 | | 1.3 | |
| | 25 | 11.2 | 23 | 1.5 | 5.2 |
| | 50 | 11.7 | | 2.5 | |
| | 100 | 12.5 | | 2.6 | |
| MurNAc-L-Ala | 100 | 10.4 | | 1.6 | |
| | 200 | 8.0 | | 1.0 | |
| FIA-Control | | 7.1; 10.7 | 10 | 1.0 | 1.0 |

[a] Average diameter (mm) of redness (erythema). [b] Ratio of antibody in the test group to that in the respective control group.

Kotani et al.[47] synthetized a variety of N-acetylmuramylpeptides (or amino acids) and peptides in order to perform detailed studies concerning the relationship between structure and activity. N-acetylmuramyl-L-alanyl-D-isoglutamine (Table V) was identified as the minimum structural entity essential for the immunoadjuvant activities characteristic of bacterial cell walls (Table VI). Consequently N-acetylmuramyl-L-alanine was not adjuvant active. The tetrapeptide portion of adjuvant-active N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine proved to be inert, at least in induction of delayed-type hypersensitivity.

The possible adjuvant activities of various analogues or diastereomers of the above N-acetylmuramyldipeptide and related compounds were studied. N-acetylmuramyl-L-alanyl-D-glutamic acid exhibited weak but definite adjuvanticity, but none of the others, including N-acetylmuramyl-L-alanyl-L-isoglutamine, N-acetylmuramyl-L-alanyl-D-glutamine and N-acetylmuramyl-L-alanyl-D-isoasparagine, had any adjuvant activity. This clearly indicated the importance of the configuration of the glutamic acid residue or its amides. i.e. the presence of the D-isoglutamine residue in the N-acetylmuramyldipeptide, for manifestation of adjuvant activities in stimulation of both antibody-mediated and cell-mediated immune responses. Neither N-acetylmuramyl-D-isoglutamine nor N-acetylmuramyl-D-alanine had any adjuvanticity[47].

### 5. *The present state and the future of this research field*

This rapid survey was sufficient to point out that very simple and small digestion or split products, which can arise in vivo from all bacteria (Gram-positive or Gram-negative) and thus are naturally widespread, belong to a group of substances able to exert an adjuvant or immunostimulating activity. However these hydrosoluble compounds were found active in the presence of FIA, and only rarely, in some special biological systems, in the absence of mineral oil.

A direct application of the hydrosoluble natural or synthetic compounds in human or animal therapeutics is hampered by this necessity of using mineral oil which is responsible for many undesirable side-reactions. In this connection Migliore et al.[48] tried quite recently to modify a series of soluble compounds by adding some easily metabolizable and non-toxic lipids (lauric or palmitic acid). The lipid conjugated substances exerted, in the guinea-pig and in the rabbit, without the need of being injected with mineral oil, significant adjuvant activity on the induction of delayed type hypersensitivity and on antibody production.

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