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Correlation Between In Vivo Anti-*Pseudomonas* and Anti-*Candida* Activities and Clearance of Carbon by the Reticuloendothelial System for Various Muramyl Dipeptide Analogs, Using Normal and Immunosuppressed Mice

ELIZABETH B. FRASER-SMITH,* RUTH V. WATERS, AND THOMAS R. MATTHEWS

Syntex Research, Palo Alto, California 94304

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A linear correlation coefficient analysis, comparing in vivo anti-infective and reticuloendothelial stimulating activity of several different analogs of *N*-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide) suggests that the macrophage is an important target cell for these immunomodulating compounds. The increase in protection against infections of *Candida albicans* or *Pseudomonas aeruginosa* in normal or immunosuppressed mice after treatment with 18 different glycopeptides was found to correlate with the degree of clearance of colloidal carbon particles from the blood by the reticuloendothelial system after treatment with the same muramyl dipeptide analogs. The compound which gave the greatest protection in all four assays was *N*-acetylmuramyl-L-α-aminobutyryl-D-isoglutamine followed by *N*-acetyl-*nor*-muramyl-L-alanyl-D-isoglutamine. Both analogs were better than the parent muramyl dipeptide. Whether macrophage stimulation alone is responsible for the anti-infective properties of these compounds has not yet been determined.

Treatment with MDP or MDP analogs has been shown to enhance resistance in mice to a variety of pathogens, including *Klebsiella pneumoniae* (5, 8, 25), *Pseudomonas aeruginosa, Candida albicans* (10, 20), *Salmonella typhimurium* (8), *Streptococcus pneumoniae* (13), *Trypanosoma cruzi* (15), and *Toxoplasma gondii* (16). The mechanism(s) underlying resistance to these bacterial, fungal, and protozoal pathogens has not been fully determined.

A number of in vitro studies have suggested that the macrophage is a target cell since MDP derivatives can stimulate several activities of these phagocytic cells, some of which have been considered to be expressions of macrophage activation. Treatment with MDP is reported to: (i) increase adherence and spreading of macrophages on glass (6); (ii) enhance phagocytic ability (11); (iii) inhibit macrophage migration (34); (iv) increase production of prostaglandin and collagenase (32), lymphocyte-activating factor (23), and superoxide anion (24); (v) enhance glucosamine uptake (29); and (vi) augment activity against neoplastic cells, including inhibition of growth (14) and direct cytolytic activity (31).

There is also evidence that in vivo treatment with MDP analogs results in macrophage stimulation and may be at least partly responsible for enhanced resistance to infection. MDP and its analogs have been shown to enhance the carbon clearance capacity of the reticuloendothelial system (RES) (30, 33) and to protect CBA mice against infection with the facultative intracellular bacterium *Listeria monocytogenes* (10). It should be noted that not all studies have found activity against *Listeria* (13), for which clearance from the body has been determined to be dependent on the macrophage (18, 19, 22).

The present study compares the anti-infective properties of 18 different MDP analogs against infections of *C. albicans* and *P. aeruginosa* in normal or immunosuppressed mice or both and demonstrates a correlation between the degree of anti-infective activity and the carbon clearance capacity of the RES.

MATERIALS AND METHODS

Compounds. Eighteen different glycopeptides were used in these studies. They were: *N*-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide, MDP); *N*-acetyl-*nor*-muramyl-L-alanyl-D-isoglutamine (*nor*MDP); *N*-acetylmuramyl-L-α-aminobutyryl-D-isoglutamine ([Abu^1]MDP); *N*-acetyl-*nor*-muramyl-L-α-aminobutyryl-D-isoglutamine ([Abu^1]*nor*MDP); *N*-acetylmuramyl-L-valyl-D-isoglutamine ([Val^1]MDP); *N*-acetyl-*nor*-muramyl-L-valyl-D-isoglutamine ([Val^1]-*nor*MDP); *N*-acetylmuramyl-L-threonyl-D-isoglutamine ([Thr^1]MDP); *N*-acetyl-*nor*-muramyl-L-threonyl-D-isoglutamine ([Thr^1]*nor*MDP); 2-(2-acetamido-2-deoxy-D-glucos-3-*O*-yl)-D-butyryl-L-alanyl-D-isoglutamine (3'-MeMDP); 2-(2-acetamido-2-deoxy-D-glucos-3-*O*-yl)-D-butyryl-L-valyl-D-isoglutamine (3'-Me[Val^1]MDP); 2-(2-acetamido-2-deoxy-D-glucos-3-

O-yl)-D-hexanoyl-L-alanyl-D-isoglutamine (3'-*n*-BuMDP); *N*-acetyl-4,6-di-*O*-acetyl-L-alanyl-D-isoglutamine (4,6-di-*O*-AcMDP); *N*-acetyl-4,6-di-*O*-acetyl-L-valyl-D-isoglutamine (4,6-di-*O*-Ac[Val^1]MDP); *N*-acetyl-4,6-di-*O*-octanoylmuramyl-L-alanyl-D-isoglutamine (4,6-di-*O*-octanoylMDP); *N*-acetyl-4,6-di-*O*-octanoylmuramyl-L-valyl-D-isoglutamine (4,6-di-*O*-octanoyl[Val^1]MDP); *N*-benzoylmuramyl-L-alanyl-D-isoglutamine ([*N*-benzoyl]MDP); *N*-hexanoylmuramyl-L-alanyl-D-isoglutamine ([*N*-hexanoyl]MDP); *N*-glycolyl-*nor*muramyl-L-alanyl-D-isoglutamine ([*N*-glycolyl]*nor*-MDP).

The *nor* prefix is used to designate compounds lacking the methyl group on the lactyl portion of the molecule.

The compounds were prepared by members of Syntex Institute of Organic Chemistry under the direction of Gordon Jones at Palo Alto, Calif. (G. H. Jones, J. J. Nestor, Jr., D. Tegg, B. Horner, T. C. Thurber, J. G. Moffatt, N. Byars, R. V. Waters, and R. Ferraresi, Abstr. 16th Natl. Medicinal Chem. Symp., June 1978, p. 107). All dose levels designated in this paper are given for *nor*MDP. The other glycopeptides were administered in doses equimolar to that given for *nor*MDP.

Animals. Female ICR-SPF mice of 18 to 20 g in weight were used for the *C. albicans* infection, and 14- to 16-g ICR-SPF females were used for the infections with *P. aeruginosa*. Mice were obtained from Charles River, Wilmington, Mass.; Simonsen Labs, Gilroy, Calif.; or Lab Supply, Indianapolis, Ind. CF-1 female mice, 6 to 8 weeks old, from Charles River were used for the carbon clearance assay.

Glycopeptide treatment. Groups of 20 mice, normal or immunosuppressed, were injected intraperitoneally (i.p.) once daily for 4 days at 96, 72, 48, and 24 h before infection. Each dose was 80 mg/kg for *nor*MDP. This treatment schedule was optimum in assuring an increase in both survival time and number of survivors for the best analogs (10). Mice treated with saline only served as virulence controls.

For the RES stimulation studies, groups of 8 to 12 mice were treated subcutaneously with a single 10-mg/kg injection of MDP or analogs in saline 24 h before the carbon clearance assay. Previous testing showed no difference in either protective activity or carbon clearance between i.p. and subcutaneous routes (10, 33). Stimulation of carbon clearance after glycopeptide treatment did not increase with additional treatments (33).

Administration of cyclophosphamide. Mice to be immunosuppressed before infection were given a single i.p. injection of 300 mg of cyclophosphamide (Cytoxan, Mead Johnson) per kg in saline at 96 h preinfection. Leukocyte counts were <1,500 cells per μl at the time of infection. The toxicity of cyclophosphamide was checked by giving animals i.p. injections without subsequent glycopeptide treatment or infection.

Bacterial infection. A human clinical isolate of *P. aeruginosa* (Pa120) was used for the bacterial infections. The organism was passed and recovered in mouse blood every 4 months and maintained at −70°C until use. *P. aeruginosa* was grown overnight in 5 ml of brain heart infusion broth at 35°C. Cells were centrifuged and suspended in saline for immunosuppressed mice or in saline containing 5% hog gastric mucin for normal mice. The challenge given to each normal mouse by i.p. injection was 7.5×10^5 colony-forming units (CFU). The challenge dose for each immunosuppressed mouse by the i.p. route was 1.1×10^6 CFU. Survivors were recorded for 48 to 66 h. The 50% lethal challenge for normal mice averaged 4×10^4 CFU (i.p. challenge in mucin) and 2×10^5 CFU (intravenous challenge in saline) for immunosuppressed mice. The data are expressed as percent increase in average survival time for glycopeptide-treated mice compared to saline controls.

Fungal infection. A human clinical isolate of *C. albicans* (Ca523) maintained at −70°C was used for the fungal infections. The organism was passed and recovered in mouse kidney every 6 months. It was prepared for challenge by suspending cells, grown overnight at 35°C on Sabouraud-Emmons slants, in physiological saline. The challenge given to each mouse intravenously was 4.5×10^6 cells in 0.2 ml of saline. Survivors were recorded for 7 to 12 days, at which time there were no surviving saline-treated control mice. The data are expressed as percent increase in average survival time for drug-treated mice compared to saline controls.

Carbon clearance assay. Clearance of carbon from the blood by the RES was measured by the method previously described (1, 33). In brief, blood samples were taken from the orbital sinus at 3- to 10-min intervals after the intravenous administration of carbon ink in saline (160 mg/kg, Pelikan C11/1431a). The optical density of each sample, after dilution in sodium carbonate, was measured at 610 nm to determine the concentration of carbon in the peripheral blood. The phagocytic index (K) was determined as the slope of the semilogarithmic plot of optical density with time. The corrected phagocytic index (α) was calculated as the cube root of K times the body weight divided by the combined weight of the liver and spleen. The results are expressed as percent increase in clearance rate (α index) for drug-treated groups compared to saline controls.

Statistical analysis. Statistical evaluation of differences in survival time between glycopeptide- and saline-treated groups was done by the Mann-Whitney U probability test (12). All mice which had not died by the end of a test were assigned a survival time of 24 h later for this comparison. Differences in the number of survivors were evaluated by the Fisher exact probability (two-tail) test (21). P values ≤ 0.05 were considered to be significant.

The difference in carbon clearance rates between each treatment group and control was assessed by the separate variance form of the t test (2).

A linear correlation coefficient test (35) was used to compare all the MDP analogs for degree of protection against infection with clearance of carbon from the blood by the RES.

RESULTS

A good correlation was seen between the degree of phagocytic stimulation after treatment with different glycopeptides, as measured by the clearance of carbon from the blood, and the protective activity of those same MDP analogs against a *C. albicans* infection (Table 1 and Fig.

1). The P value for significance was <0.001 when all 18 glycopeptides were compared by linear correlation analysis. The most active analog, producing the greatest increase in either survival time or carbon clearance over saline-treated controls, was [Abu[1]]MDP. [Abu[1]]-*nor*MDP, *nor*MDP, and 3'-MeMDP also produced good responses in both assays. Of the 18 analogs tested, 13 produced a statistically significant increase in both survival time and number of survivors of mice at the time when all saline control animals had died.

A statistically significant correlation (P = 0.025) was also seen between carbon clearance and protective activity against a *P. aeruginosa* infection in normal mice (Table 1). The linear correlation between the two parameters for the 18 glycopeptides was not as strong as with *C. albicans*. As with *Candida*, the best protective activity against *P. aeruginosa* was seen with [Abu[1]]MDP. *nor*MDP also gave a good response. Only 7 of the 18 analogs produced a statistically significant increase in both survival time and number of survivors against this pathogen, whereas 13 had done so against the *C. albicans* infection.

The linear correlation seen against a *P. aeruginosa* infection in normal mice was also apparent in mice immunosuppressed with cyclophosphamide. The P value comparing carbon particle clearance and protective activity was 0.018 when all 18 MDP analogs were analyzed. [Abu[1]]MDP and *nor*MDP continued to provide the best protective activity in this third infection model. Only 4 of the 18 analogs produced a statistically significant increase in both survival time and number of survivors in immunocompromised animals.

For further comparison, there was no significant increase or decrease in the extent of carbon clearance to anti-infective activity when we compared the 11 of the 18 analogs in which the amino acid side chain was altered in all three infection models (P < 0.001, 0.03, and 0.015 with *C. albicans* and *P. aeruginosa* in normal or *Pseudomonas* in immunosuppressed mice, respectively).

DISCUSSION

Evidence obtained with the present 18 MDP analogs suggests that a nonspecific enhancement of phagocytic cell function by glycopeptide is involved in the mechanism(s) by which these

TABLE 1. Linear correlation between average survival time and clearance of colloidal carbon from blood by the RES

MDP analog	% Increase in carbon clearance (α index)[a]	% Increase in avg survival time[a] with:		
		C. albicans in normal mice	*P. aeruginosa* in:	
			Normal mice	Immunosuppressed mice
MDP	16	57[b]	15	24
*nor*MDP	19 ± 5	59[b]	107[b]	64[b]
[Abu[1]]MDP	25 ± 5	68[b]	128[b]	64[b]
[Abu[1]]*nor*MDP	22 ± 4	60[b]	60[c]	44[b]
[Val[1]]MDP	15 ± 5	58[b]	39[c]	52
[Val[1]]*nor*MDP	9 ± 3	14	60[c]	0
[Thr[1]]MDP	0	16	18	−8
[Thr[1]]*nor*MDP	0	20	25	−12
3'-MeMDP	22 ± 4	66[b]	43[b]	44
3'-Me[Val[1]]MDP	0	0	33[c]	28
3'-*n*-BuMDP	0	6	31[c]	40
4,6-di-*O*-AcMDP	16 ± 7	58[b]	43[c]	−8
4,6-di-*O*-Ac[Val[1]]MDP	12 ± 5	31[b]	64[b]	20
4,6-di-*O*-octanoylMDP	0	43[b]	49[b]	16
4,6-di-*O*-octanoyl[Val[1]]MDP	12 ± 6	51[b]	7	44
[*N*-benzoyl]MDP	5 ± 5	37[b]	30[c]	28
[*N*-hexanoyl]MDP	11 ± 4	61[b]	46[b]	−4
[*N*-glycolyl]*nor*MDP	15 ± 4	39[b]	61[b]	52[b]
Linear correlation coefficient P value		0.001	0.025	0.018

[a] MDP analog-treated mice compared to saline-treated control.

[b] $P \leq 0.05$ for both average survival time (Mann-Whitney U probability test) and number of survivors (Fisher exact probability), MDP analog compared to saline control. Values left blank were not considered significant.

[c] $P \leq 0.05$ for average survival time only.

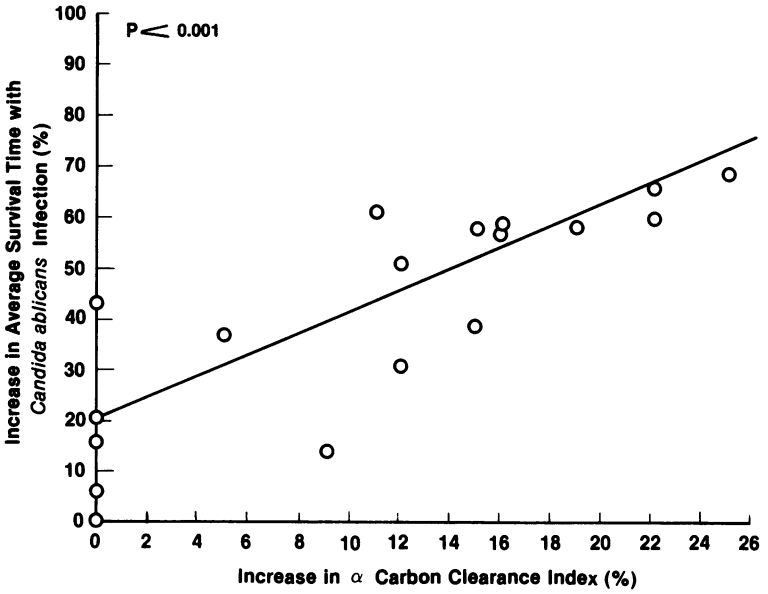

FIG. 1. Linear correlation between anti-*Candida* activity and clearance of colloidal carbon particles from the blood by the RES for 18 different MDP analogs after treatment in mice. Data are expressed as percent increase for drug-treated groups compared to saline controls.

compounds protect against infection. A linear correlation was found between the degree to which different MDP analogs accelerate clearance of colloidal carbon from the circulation of mice with the anti-infective activity of those same compounds against either *C. albicans* or *P. aeruginosa*. The statistical correlation was strongest with the *C. albicans* infection, the *P* value being <0.001, whereas it was ~0.02 with a *P. aeruginosa* infection in either normal or immunosuppressed mice. This information is consistent with data from other studies; recent papers, which have indicated that the macrophage is an important target cell for the biological effects of MDP, are documented in the introduction. Much of this work was done with in vitro or ex vivo models. The present results are one of the few examples of data obtained with in vivo studies.

The fact that a linear correlation was seen between carbon clearance and anti-infective activity in both normal mice and mice immunosuppressed with cyclophosphamide also suggests the involvement of the macrophage as a target cell. Macrophage production and function were studied by Buhles and Shifrine (3, 4) in mice receiving cyclophosphamide at doses used in the present experiments. A level of macrophage activity was found 2 to 3 days after cyclophosphamide treatment that was relatively cyclophosphamide resistant in both immunosuppressed and adjuvant-treated, immunosuppressed mice. Cyclophosphamide treatment resulted in a monocytopenia and reduced bone marrow monocyte production which lasted 2 to 3 days, followed by a monocytosis while other leukocyte components often remained low. Tissue macrophage numbers varied less than the numbers of granulocytes or lymphocytes after treatment. Collected macrophages appeared to be functionally normal when tested for glass adherence, phagocytosis, and intracellular digestion of live *Candida*. Consequently, they postulated that the heightened level of macrophage activity seen after adjuvant treatment with *Mycobacterium bovis* BCG may be a cause of the nonspecific protection observed against infection during immunosuppression with cyclophosphamide. The present results are in agreement with this concept. The fact that linear correlations of the same magnitude were observed between anti-*Pseudomonas* activity in either immunosuppressed or normal mice and the clearance of carbon by the mononuclear phagocytes of the RES suggests that the macrophage is relatively cyclophosphamide resistant. It strengthens the concept that the macrophage is a target cell of MDP analog treatment.

It is not possible to determine from the present experiments whether or not the macrophage is the principal or sole cell type which is stimulated by MDP analogs. A possible role for lymphocyte-mediated activation of macrophages cannot be ruled out completely. MDP has been

reported to modify T-cell-dependent responses (17, 27), as well as display mitogenic activity on B-cells (7, 26, 28).

Although the present correlations indicate that macrophages were stimulated after glycopeptide treatment, it is not known whether protection is mediated by a cytotoxic or cytostatic mechanism. Fidler et al. (9) found that the injection of MDP in saline did not render alveolar macrophages of mice tumoricidal, whereas MDP injected in liposomes did activate alveolar macrophages to become tumoricidal against B16-BL6 melanoma cells. It is possible that treatment with free MDP analogs stimulates macrophages only at a microbistatic level. In this case, the pathogenic organism would be engulfed and yet maintain its viability. Protection would be achieved by alterations in metabolism, caused by the macrophage, resulting in a slowing of the pathogen's growth or a reduction in its virulence or both. The pathogen could conceivably be contained by such mechanisms until the normal immune response of the animal, caused by exposure to specific antigens of the invading pathogen, became functional. Further work is needed to elucidate under what conditions MDP treatment may produce macrophages which possess microbicidal activity.

In summary, although the exact mechanism of protection after glycopeptide treatment is not known, the fact that positive correlations between carbon clearance and anti-infective activity have been found in both *C. albicans* and *P. aeruginosa* in in vivo infection models strengthens the concept that the macrophage is a target cell.

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