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# Depressed Chemiluminescence Response by Influenza Virus is Enhanced after Conjugation of Viral Subunits to Muramyl Dipeptide

K. NOEL MASIHI,[1]* WERNER LANGE,[1] BEATE ROHDE-SCHULZ,[1] LOUIS CHEDID,[2] AND MICHEL JOLIVET[2]

*Robert Koch Institute, Federal Health Office, D-1000 Berlin 65, Federal Republic of Germany,[1] and Institut Pasteur, Immunothérapie Expérimentale, Paris, France[2]*

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**The effect on respiratory burst of murine spleen cells after in vitro exposure to influenza virus, subunits, or subunits conjugated to muramyl dipeptide (MDP) was studied by luminol-dependent chemiluminescence (CL) in response to stimulation by zymosan. CL induced by infectious influenza A virus was depressed but could be elevated to normal levels when MDP was added together with a low, but not with a high, dose of the virus. Profound depression of CL was induced by high doses of influenza A/Brazil, A/Bangkok, and B/Singapore subunits. The same amounts of viral subunits conjugated to MDP restored or even enhanced the CL responses of spleen cells from BALB/c and C57BL/6 mice. Splenic cells from BALB/c mice generated higher levels of CL than did cells from C57BL/6 mice.**

A variety of virus infections are associated with a dysfunction of the phagocytic cells. Influenza viruses have been most widely studied in this respect and were demonstrated to diminish phagocytosis, intracellular killing, and chemotaxis in humans (17, 19), guinea pigs (29), chinchillas (1), rats (25), and mice (15). Impaired phagocytic cell function was observed with other viruses including Sendai virus (13), respiratory syncytial virus (8), infectious bovine rhinotracheitis virus (11), mumps virus (22), herpes simplex virus (24), and cytomegalovirus (26).

The process of phagocytosis generates activated oxygen metabolites which are associated with the emission of light (2). Luminol-dependent chemiluminescence (CL) is a sensitive assay for monitoring the respiratory burst of phagocytic cells (10). Infectious influenza A and B viruses can considerably depress CL (1, 21). Commercially available inactivated trivalent influenza whole virus, split virus, and subunit vaccines also depress CL (21).

Phagocytic cells appear to be among the target cells for the immunopotentiating activity of biological response modifiers like synthetic muramyl dipeptide (MDP), which is the minimum structure essential for bacterial adjuvanticity (18). Superoxide anion generation (14, 23) and zymosan-induced CL (20) can be enhanced by MDP. We considered it of interest to study whether a combination of viral antigens with MDP could retard or even reverse the virus-induced depression of the phagocytic cells. In the present study, we used a luminol-dependent CL assay to investigate the effects of influenza virus subunits coupled to MDP on the respiratory burst of splenic cells from mice.

## MATERIALS AND METHODS

**Animals.** Six-week-old BALB/c mice were obtained from the specific-pathogen-free colony maintained by the Bundesgesundheitsamt, Berlin, Federal Republic of Germany. C57BL/6 mice were purchased from Zentralinstitut für Versuchstiere, Hanover, Federal Republic of Germany.

**Preparation of influenza virus and subunits.** Influenza A/Brazil/11/78 (H1N1), A/PR/8/34 (H1N1), A/Bangkok/1/79 (H3N2), and B/Singapore/222/79 viruses were grown in the allantoic cavities of 11-day-old embryonated eggs. The freshly harvested allantoic fluid was centrifuged lightly (2,000 × *g* for 15 min) to remove erythrocytes and debris. Influenza A/PR/8/34 virus was used without further treatment. The clarified preparations of infectious virus were concentrated by centrifugation in a Beckman 19 rotor at 18,000 rpm for 1 h at 4°C. The virus concentrate was warmed to 20°C, and a 0.25% solution of *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide (E. Merck AG, Darmstadt, Federal Republic of Germany) was added at a virus-to-*N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide ratio of 9:1. The mixture was kept at 20°C for 1 h and then centrifuged for 40 min at 25,000 rpm in a Beckman 50-1 rotor. The supernatant containing hemagglutinin (HA) and neuraminidase spikes was dialyzed for 3 days against a large volume of sterile phosphate-buffered saline with three changes of the buffer. The HA antigen content of influenza subunits was determined by single radial immunodiffusion assay (32) or by an automated fluoroimmunoassay (FIAX system) based on measurement of the amount of unconsumed antibody after reaction with standardized amounts of antigen used to calibrate a standard curve (16).

**Conjugation of influenza subunits to MDP.** Influenza virus subunits were conjugated to MDP–L-lysine by the glutaraldehyde method (5). Viral subunits representing 825 μg of HA and 7 mg of MDP-lysine were added to 0.1 M sodium bicarbonate. A 25% solution of glutaraldehyde was slowly added to a final concentration of 2.6 mM, and the reaction was allowed to proceed under magnetic stirring for 4 days at 20°C. Nonreactive reagents were eliminated from the conjugate by exhaustive dialysis against phosphate-buffered saline. Analysis of the conjugate showed that 51, 92.6, and 55.6 μg of MDP-lysine, respectively, was coupled to approximately 100 μg of A/Brazil, A/Bangkok, and B/Singapore subunits.

**Luminol-dependent CL assay.** A CL assay with spleen cell suspensions has been described previously (20). After measurement of the background for 3 min, CL was generated by adding the viral preparations, with or without MDP, followed immediately by 10 μl of nonopsonized zymosan (Sigma, Munich, Federal Republic of Germany) at a concen-

* Corresponding author.

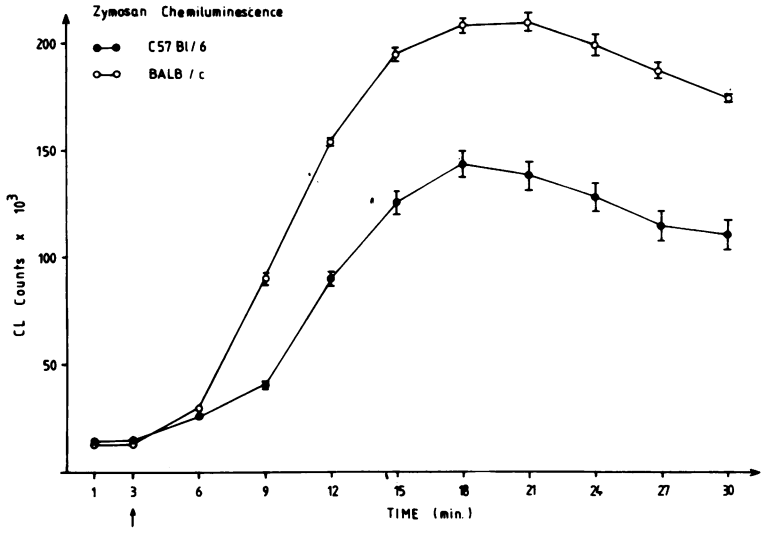


FIG. 1. Zymosan-induced CL response of BALB/c and C57BL/6 spleen cells treated in vitro with murabutide. The results are expressed as mean ± standard error of three experiments.

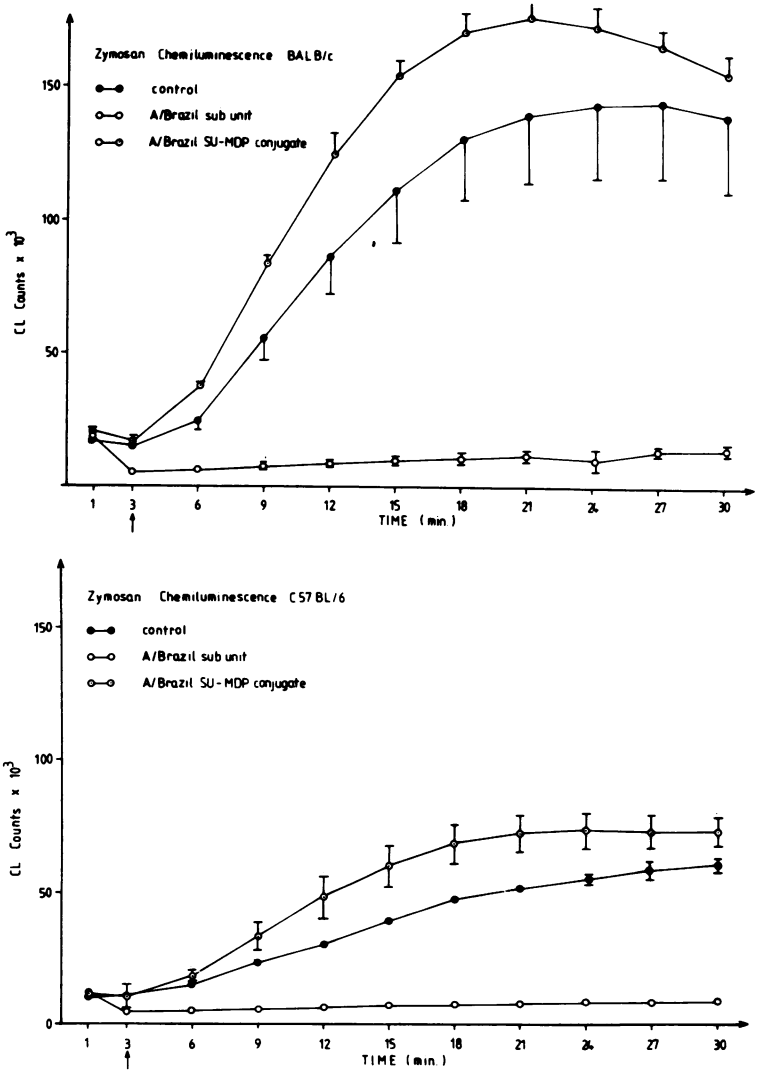


FIG. 2. Zymosan-induced CL response of spleen cells treated in vitro with influenza A/Brazil subunits alone or conjugated to MDP-lysine. The results are expressed as mean ± standard error of three experiments.

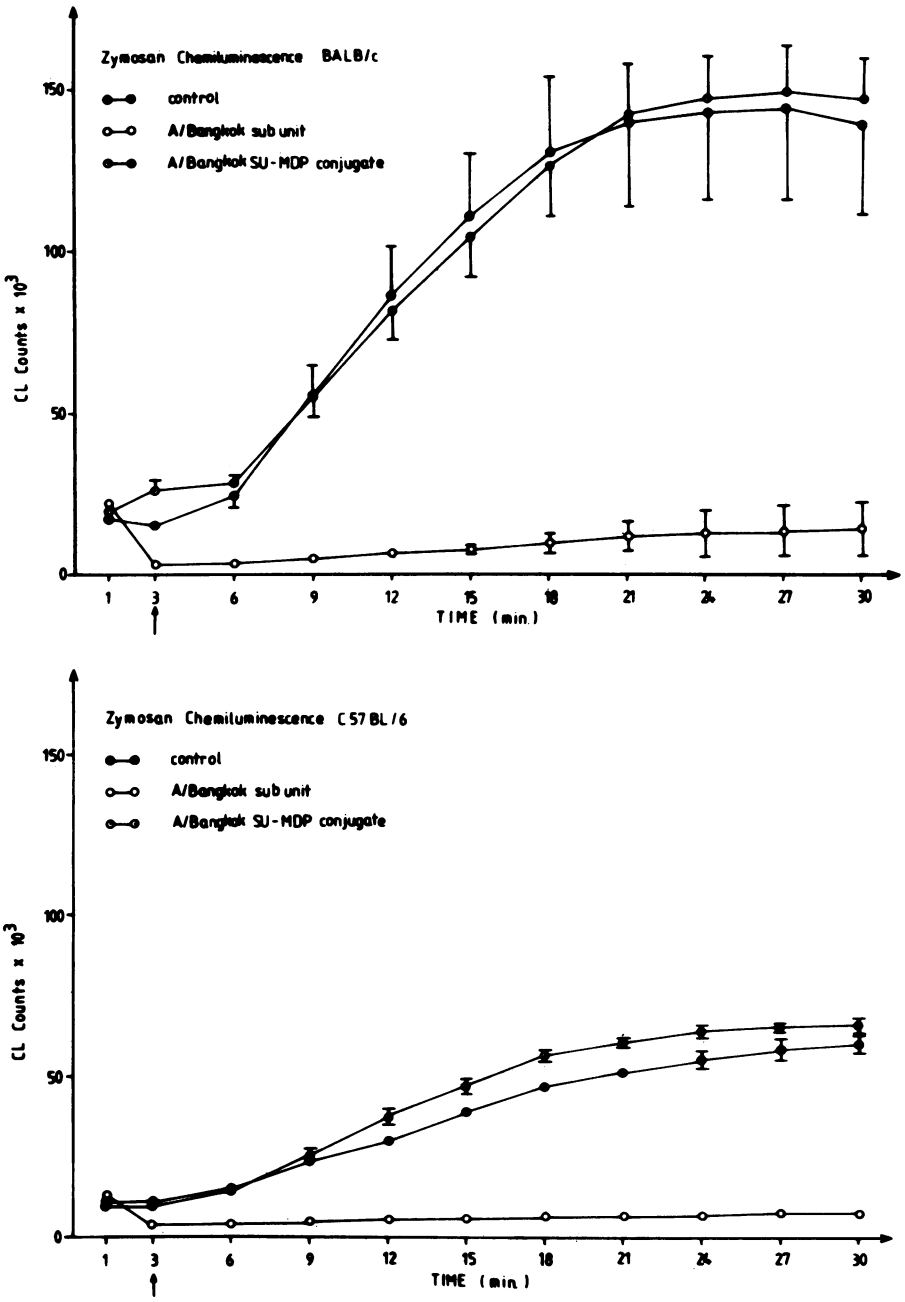


FIG. 3. Zymosan-induced CL response of spleen cells treated in vitro with influenza A/Bangkok subunits alone or conjugated to MDP-lysine. The results are expressed as mean ± standard error of three experiments.

tration of 50 mg/ml. CL measurements in six-channel Biolumat (Berthold, Wildbad, Federal Republic of Germany) were continuously monitored on a programmed Apple II computer.

## RESULTS

All of the following experiments were performed by measuring the zymosan-stimulated CL of murine splenic cells.

**Effect of infectious influenza A virus and MDP on BALB/c cells.** In initial exploratory experiments, 100 or 1,000 HA units (HAU) of infectious influenza A/PR/8/34 virus was added to spleen cells of BALB/c mice. The results presented in Table 1 show that the zymosan-induced CL response was depressed by the virus. A significantly higher CL activity was stimulated when MDP was added together with the infectious virus than when the virus alone was added. CL activity was higher than that of the zymosan controls when 100 but not when 1,000 HAU of infectious virus was used in combination with MDP. Generation of CL by both doses of virus plus MDP was lower than that stimulated by MDP alone, but again the CL activity was higher with 100 than with 1,000 HAU of infectious virus.

**Effect of murabutide on BALB/c and C57BL/6 cells.** To determine whether there were differences in the CL responses of MDP, spleen cells from genetically different

TABLE 1. Zymosan-induced CL of spleen cells treated with infectious influenza virus and MDP

| Treatment | Integrated total CL counts/30 min ($10^4$) |
|---|---|
| Zymosan[a] | 196.2 ± 6.7[b] |
| Zymosan + 100 HAU of influenza virus[c] | 175.5 ± 5.0 |
| Zymosan + 100 HAU of influenza virus + 100 μg of MDP | 232.8 ± 8.8 |
| Zymosan + 1,000 HAU of influenza virus | 114.4 ± 1.8 |
| Zymosan + 1,000 HAU of influenza virus + 100 μg of MDP | 162.4 ± 3.0 |
| Zymosan + 100 μg of MDP | 258.1 ± 15.1 |

[a] A 10-μl portion of a 50-mg/ml suspension of zymosan was added to the spleen cell suspensions of BALB/c mice.
[b] Results are expressed as mean ± standard error of six replicate cultures.
[c] HAU of infectious A/PR/8/34 (H1N1) influenza virus.

BALB/c and C57BL/6 mice were used. A nonpyrogenic analog of MDP, murabutide (7), was added to spleen cells. Significant differences were observed in the zymosan-induced CL responsiveness of the two mouse strains (Fig. 1). Spleen cells from BALB/c mice generated higher levels of CL than did spleen cells from C57BL/6 mice.

**Effect of influenza A and B virus subunits and subunit-MDP conjugates on BALB/c and C57BL/6 cells.** Viral subunits alone or coupled to MDP were added to suspensions of spleen cells from untreated mice at a dosage of 10 μg of HA. Preliminary experiments showed that this dose of subunits exerted a highly inhibitory effect on the CL activity of spleen cells. A marked depression of CL response was induced in both the mouse strains by A/Brazil subunits (Fig. 2). In contrast, a dramatic stimulation of CL was generated by the A/Brazil subunit-MDP conjugate. The CL activity of the A/Brazil subunit-MDP conjugate on BALB/c and C57BL/6 cells even surpassed that induced by zymosan alone. Subunits of A/Bangkok virus strongly inhibited CL response in both the strains, but the A/Bangkok subunit-MDP con-

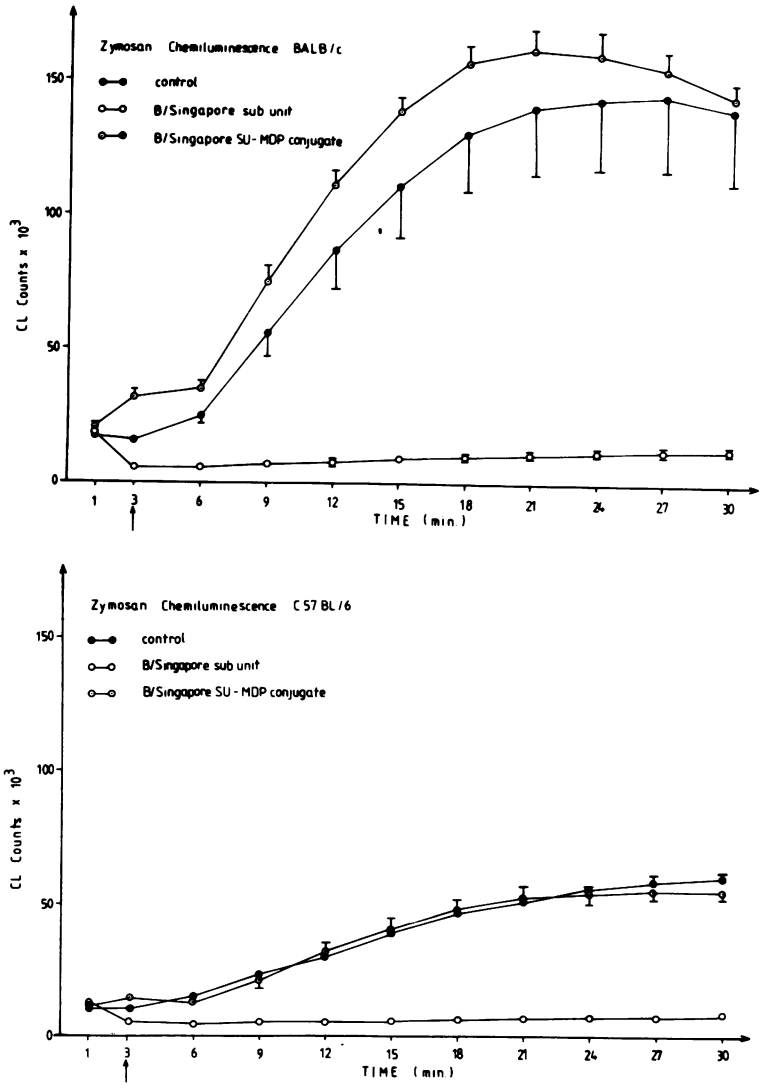


FIG. 4. Zymosan-induced CL response of spleen cells treated in vitro with influenza B/Singapore subunits alone or conjugated to MDP-lysine. The results are expressed as mean ± standard error of three experiments.

jugate stimulated CL activity which was similar to that generated by zymosan alone (Fig. 3). Influenza B/Singapore subunits depressed CL to a similar extent as did influenza A subunits (Fig. 4). Addition of the B/Singapore subunit-MDP conjugate stimulated a CL activity which was higher in BALB/c but similar to that of zymosan controls in C57BL/6 cells.

## DISCUSSION

Clearance of some bacteria is closely associated with the phagocytic cells (30). The influenza virus-induced depression of CL is limited to unseparated and phagocyte-enriched populations, but no apparent effect is found in the lymphocyte-enriched populations (21). Depression of CL by influenza is not restricted to infectious virus, but extends to commercially available inactivated influenza virus vaccines (21). Virus-induced dysfunction of the phagocytic cells can predispose a susceptible host to bacterial superinfection (1, 8, 13, 17, 25). Excessive mortality from bacterial pneumonia is known to occur during influenza epidemics and is actually used as one of the nonvirologic indicators for influenza surveillance (12). Procedures for retarding the virus-induced impairment of phagocytic cell function could have important practical implications.

Immune responses induced by a variety of natural and synthetic antigens have been enhanced by MDP. The production of antibodies to influenza virus (4, 31) and hepatitis B surface antigens (6) was increased by MDP or analogs. The results presented in this study demonstrate that the generation of respiratory burst in mouse spleen cells as measured by zymosan-induced CL is impaired by influenza virus but can be substantially improved by combining the virus with MDP. The moderate depression of CL induced by 100 HAU of infectious influenza virus could be completely overcome when the virus and MDP were added together to the cells. A higher dose, of 1,000 HAU of infectious influenza virus, induced a greater depression of the CL response, which remained below the control zymosan values even when MDP was present. This observation indicated that a more efficient mode of presenting MDP than simple addition would be necessary. Direct chemical conjugation of the viral subunits to MDP enhanced the immunostimulatory activity of the preparations so that the profound inhibition induced by high doses of influenza subunits could be overcome. In a previous study, strong antiviral responses against coliphage MS-2 could be induced when MDP was conjugated, but not when it was mixed, with a synthetic fragment of the virus coat protein attached to a synthetic polymeric carrier (3).

Macrophages appear to be among the target cells for the immunostimulating activities of MDP, and the generation of zymosan-stimulated CL can be enhanced by MDP and analogs (20). The configuration of the viral subunits coupled to MDP may have enabled an efficient presentation of the conjugated MDP, resulting in an amplification of the CL response by direct effect on the target cell.

Several properties of influenza A/Brazil, A/Bangkok, and B/Singapore subunits were altered after conjugation with MDP-lysine. The capacity of influenza A conjugates to agglutinate erythrocytes was significantly reduced. It is of interest that despite this the conjugation not only restored the CL of all the three influenza subunits but also enhanced nonspecific resistance against *Klebsiella pneumoniae* infection after pretreatment with A/Brazil or B/Singapore conjugates. Furthermore, intranasal pretreatment of mice with B/Singapore conjugate even induced resistance against aerosol infection with heterologous A/PR/8 influenza virus (M. Parant, N. Masihi, W. Lange, W. Brehmer, F. Parant, M. Jolivet, and L. Chedid, submitted for publication). Whether there exists a relationship between these various activities remains to be investigated.

A further finding to emerge from the present studies is that the CL responsiveness of different strains of mice to MDP can vary. Murabutide stimulated higher zymosan-induced CL activity in spleen cells of BALB/c mice than in those of C57BL/6 mice. The CL responses to influenza virus subunits conjugated to MDP were also greater in BALB/c mice than in C57BL/6 mice. These results extend the observations of mouse-strain-dependent responsiveness of MDP. In previous studies, the mitogen responsiveness of spleen cells to MDP was found to be high with BALB/c but low with C57BL/6 mice (9). Secondary antibody responses to bovine albumin, the terpolymer of L-glutamic acid, L-alanine, and L-tyrosine, and bacterial α-amylase were greatly enhanced in BALB/c mice but not in C57BL/10 mice (27). In another study, macrophage-mediated cytotoxicity by MDP was augmented in BALB/c but not in C57BL/6 mice (28). Despite lower response in C57BL/6 mice, influenza subunits conjugated to MDP could stimulate levels of CL which were higher than (influenza A) or similar to (influenza B) those induced by zymosan alone.

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