COMBINED PROTECTIVE EFFECT OF AN IMMUNOSTIMULATORY BACTERIAL PREPARATION AND RIMANTADINE IN EXPERIMENTAL INFLUENZA A VIRUS INFECTION

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Summary. – The protective effect of an immunostimulatory bacterial preparation, cytoplasmic membranes of *Escherichia coli* WF' stable protoplast type L-forms (CM) alone and in combination with the selective antiviral drug rimantadine was evaluated in experimental influenza A/Aichi/2/68 (H3N2) virus infection in mice. In sublethal infection, CM administered intraperitoneally (i.p.) 7 days before virus exposure in a single dose of 25 mg/kg did not reduce significantly the virus lung titers. In lethal infection, CM applied in the same way weakly reduced the mortality rate. The combined application of CM with rimantadine resulted in synergistically increased protection, determined on the basis of virus lung titers, lung consolidation, mortality rates, protective indices, and survival times.

Key words: experimental influenza infection; bacterial cytoplasmic membranes; rimantadine; combined protective effect

Introduction

Influenza continues to be a major cause of high morbidity and significant mortality both in humans and domestic animals. Rimantadine, an analog of amantadine has well documented prophylactic (Dolin *et al.*, 1982) and therapeutic (Van Voris *et al.*, 1981) activity in uncomplicated influenza A virus infection after oral administration. Rimantadine has no marked antiviral activity or therapeutic effectiveness in established influenza and no specific therapy of proven value currently exists for severe influenza infection. Some adverse effects of rimantadine have also been reported. Herrmann *et al.* (1990) found that following a rimantadine treatment some depression of cytotoxic T-lymphocyte and antibody responses in a mouse model ocurred. In addition, a development of viral resistance to rimantadine has been identified as a problem in the use of this drug (Hayden *et al.*, 1992). Obviously, a need for effective therapy of influenza virus infection persists.

Influenza is one of the often cited examples of viral infections associated with immunosuppression (Roberts and Domurat, 1989). It has been suggested that the immune responses to influenza infection more accurately reflect an immunofocusing of the host defense capabilities – virus-non-specific responses are depressed, while virus-specific systemic, local and recruited responses are developed (Roberts, 1988). Therefore the control of influenza infection can be improved by the use of appropriate immunomodulators and selective antiviral agents can be used in combination with immunomodulatory substances.

We have reported previously that CM induced a marked immunostimulating effect on the functions of the peritoneal macrophages (Ma) in intact experimental animals, as

Abbreviations: CM = cytoplasmic membranes of *Escherichia coli* WF' stable protoplast type L-forms; CAM = chorioallantoic membranes; ELISA = enzyme-linked immunosorbent assay; i.p. = intraperitoneally; LPS = lipopolysaccharide; Ma = macrophage; PBS = phosphate-buffered saline; PHA = phytohaemagglutinin; p.i. = post infection; PMN = polymorphonuclear lymphocyte

well as on the proliferation of the antigen-binding T-lymphocytes, T-helper lymphocytes, B-lymphocytes and NK-cells (Ivanova, 1986).

The present study was undertaken to determine the protective effect of the immunostimulating bacterial preparation CM alone and in combination with the selective antiinfluenza drug rimantadine in experimentally induced influenza virus infection in mice.

Materials and Methods

CM were isolated from the stable protoplast L-forms of *E. coli* WF' according to a method described previously (Ivanova, 1986). Briefly, the cells were cultivated in trypic soy broth (Difco) pH 7.8 supplemented with 1% yeast extract (Difco), 10% inactivated horse serum (Sigma) and 1000 U/ml penicillin at 37°C for 24 hrs. The cells were sedimented by centrifugation, washed 3 times with 0.05 mol/l Tris pH 7.8, suspended in the lysing solution (50 μg/ml DNAse (Fluka), 0.01 mol/l $MgCl_2$, 0.05 mol/l Tris pH 7.8) and incubated at -18°C for 24 hrs. The lysed cells were washed 3 times by centrifugation at 0°C and the obtained CM were lyophilized. The purity of CM preparations was controlled by phase contrast and electron microscopy. For the antiviral experiments *in vitro* the suspensions of CM were made *ex tempore* in a cell culture medium and for the experiments *in vivo* in physiological saline.

Rimantadine hydrochloride was from Hoffman-La Roche Inc., Nutley, NJ.

Virus. Influenza virus A/Aichi/2/68 (H3N2) adapted to mouse lungs was obtained from Institute of Microbiology, Sofia.

Tissue culture. Chorioallantoic membranes (CAM) were prepared from 11-day-old fertile hen eggs according to Maltzeva *et al.* (1973).

Mice. Male and female 16 – 18 g ICR mice were obtained from the Experimental Animal Station of the Bulgarian Academy of Sciences in Slivnitza, Sofia.

Influenza virus infection of mice was induced under ether anaesthesia by intranasal inoculation of the virus which caused haemorrhagic pneumonia. For sublethal infection, an inoculum of 1 LD_{50} of the virus in 0.05 ml of physiological saline per mice was employed. To cause lethal infection, mice were infected with 5 –10 LD_{50} of the virus in the same volume.

Virus titration. Mouse lungs (5 for each determination) taken on day 5 p.i. were homogenized to a 10% suspension phosphate-buffered saline (PBS) and ten-fold dilutions of the supernatant after centrifugation were assayed for infectious virus in CAM cultures by the haemagglutination of hen erythrocytes (Reed and Muench, 1938). Virus titers were expressed as log $TCID_{50}$/0.5 ml.

Lung consolidation. Mouse lungs (5 for each determination) taken on day 5 p.i. were examined and scored 0 (normal), 1 (25% consolidation), 2 (50% consolidation), 3 (75% consolidation), 4 (100% consolidation).

Experimental design. In *sublethal infection*, CM was inoculated i.p. to mice 7 days before virus infection in the dose of 25 mg/kg in 0.2 ml of physiological saline. The dose of 25 mg/kg was previously determined as optimal concerning the immunostimulating and toxic properties (Ivanova, 1986). The virus titers in mouse lungs were estimated at days 1,3,5,7,10,14 and 21 p.i. In *lethal infection*, various experimental design was used to evaluate the efficacy of CM against influenza infection in mice. (1) Mice were inoculated with CM 24 hrs before or 2, 24, 48 and 72 hrs after virus infection. (2) Mice were inoculated with CM 7 days before virus infection to determine the dependence of the effect of CM on the virus inoculum. (3) Mice were inoculated with CM 1 – 8 days before infection to determine the dependence of the protective effect on the time of application of CM. (4) Mice were inoculated with CM 7 days before infection and treated with rimantadine in total dose of 10, 20 and 40 mg/kg in saline, applied orally 24 hrs before or 2, 24, 48 and 72 hrs after virus infection.

There were groups of experimental animals infected and treated only with CM, treated only with rimantadine and treated with both substances to determine the combined protective effect. Each experimental group consisted of 20 animals. Two additional groups of five animals from each experimental group were killed on day 5 and their lungs were assayed for virus titer consolidation. Mice were observed for death daily for 14 days. Toxicity controls (5 mice per combination) were run in parallel; acute and chronic lethal toxicity was followed (Ivanova *et al.*, 1993).

Evaluation of results. The protective effect of CM, rimantadine and their combination on the experimental infection was estimated by the decrease of mortality (%), protective index (PI,%), reduction of lung virus titers ($TCID_{50}$/0.5 ml), and lung consolidation (score) and prolongation of mean survival time (days) according to Ilienko (1977). PI was calculated from the equation

$$PI = \frac{PR - 1}{PR} \times 100$$

where PR (protective ratio) is $M_{control}/M_{experiment}$ and M is mortality.

The results are the mean values from 3 – 4 independent experiments. The reduction of mortality rates as compared to placebo controls was evaluated using the χ^2 analysis. The *t*-test was employed to analyze differences in survival times and virus titers in lungs. The Wilcoxon test was used to compare score determinations. Standard deviations were determined of the 95% confidence interval.

The combined effect was evaluated according to Webb (1966). The effect of the combination ($E_{1,2} = PI_{1,2}/100$) and the effects of the individual substances ($E_1 = PI_1/100$ and $E_2 = PI_2/100$) are related in the equation $E_{1,2} = E_1 + E_2 - E_1 \times E_2$; the combined effects is synergistic if $E_{1,2}$ is higher, additive if $E_{1,2}$ is equal and antagonistic if $E_{1,2}$ is lower than $E_1 + E_2 - E_1 \times E_2$.

Results

In the initial experiment run in mice to evaluate the effect of CM on the experimental influenza infection it was found that in a dose of 25 mg/kg the substance was not effective (PI <12%) when applied i.p. according to a schedule, rutinely used in our laboratory for the screening of antiviral

Table 1. Dependence of the protective effect of CM in experimental influenza infection in mice on the time of inoculation of CM

Time of inoculation of CM (days before infection)	Mortality ± SD (%)	Protective index (%)	Mean survival time (days)
Virus control	50.0±12.6		10.6
8	19.0±9.8	62.0	12.4
7	12.3±8.3	75.5	12.9
6	35.0±12.1*	29.6	10.6
5	20.8±10.1	58.0	11.5
4	35.0±12.1*	29.6	8.6
3	21.0±10.4	57.5	11.5
2	45.6±12.6*	8.8	10.0
1	38.5±12.3*	23.0	10.2

CM (25 mg/kg) was inoculated i.p. into mice 1-8 days before infection with 5 LD_{50} of the virus. 20 mice per group were used. The results are the means of 3 experiments. *The difference with virus control is not significant ($P < 0.05$).

agents (lethal infection (1), see Materials and Methods). The mortality of the virus control group was 60 – 70%. A weak protective effect (PI = 50%) was observed when CM was applied i.p. 7 days prior to infection (lethal infection (2), see Materials and Methods). In order to evaluate a possible selective virus-inhibitory activity of CM, the effect of CM was tested on the reproduction of two strains of influenza virus, A/chicken/Germany/34 strain Rostock (H7N1) and A/chicken/Germany/27 strain Weibridge (H7N7), in cultures of chick embryo fibroblasts. When applied in the maximal tolerated concentration of 0.125 mg/ml, CM did not reduce significantly the expression of viral haemagglutinin (estimated by enzyme-linked immunosorbent assay (ELISA) (Belshe *et al.*, 1988) on the infected cell surface as a measure of virus growth (13 – 23.6% inhibition). In all experiments, rimantadine (1 μg/ml) was used as a positive control – it caused 80 – 90% inhibition of virus replication (data not shown).

In a sublethal influenza infection in the murine model, CM did not reduce significantly the virus infectious titers in mouse lungs, determined at days 1, 3, 5, 7, 10, 14 and 21 p.i., although the tendency of reduction was consistently preserved at days 1, 3, 5 and 7 p.i.

CM did not reduce significantly the mortality of mice in a lethal infection when applied as a typical immunostimulator in a single dose of 25 mg/kg i.p. 7 days before virus inoculation with exception of a mild infection (mortality of virus control = 50%, PI = 75.5%) (data not shown). Thus the virus inoculum was an important variable in determining the protective effect of CM. The optimal time of application of CM to mice for a significant protection was 7 days before virus infection (lethal infection with 5 LD_{50} per mouse) (Table 1).

When CM was applied to lethaly infected mice (10 LD_{50} per mouse) in combination with the selective antiinfluenza drug rimantadine, the combined protective effect was markedly increased as compared to either single intervention (Table 2). Rimantadine was used in three different doses (10, 20 and 40 mg/kg) which protected mice to various degree. The protection caused by CM applied alone was not significant; rimantadine in concentration of 10 mg/kg, applied alone, was marginally effective. Rimantadine in doses of 20 and 40 mg/kg significantly reduced the mortality, lung virus titers and lung consolidation. The combined application of rimantadine in all three concentrations with CM lead to a marked increase of protection – all three com-

Table 2. Combined protective effect of CM and rimantadine on experimental influenza infection in mice

Experimental group	Dose (mg/kg)	Mortality ±SD (%)	Protective index (%)	Combined effect ($E_{1,2}$)	Virus lung titer log $TCID_{50}$/0.5ml±SD	Lung consolidation[a]	Mean survival time (days)
Virus control		85.0±9.0			6.6±0.5	4.0±0.5	8.6
CM	25	78.3±12.6*	7.9	–	6.5±0.4*	4.0±0.5	8.6
R1	10	75.0±11.0*	11.8		6.2±0.3*	4.0±0.2	10.3
CM+R1	25+10	35.0±12.1	59.5	synergistic	5.1±0.6	1.8±0.3	12.4
R2	20	41.6±12.4	52.3		4.7±0.5	1.9±0.3	12.0
CM+R2	25+20	10.0±7.6	88.8	synergistic	3.1±0.7	1.0±0.4	13.8
R3	40	11.0±7.9	62.0		3.5±0.6	0.9±0.2	14.0
CM+R3	25+40	10.0±7.6	88.8	additive	3.5±0.6	0.7±0.1	14.2

R1, R2, R3 = different concentrations of rimantadine (total dose). CM was inoculated i.p. to mice 7 days before infection with 10 LD_{50} of the virus. Rimantadine in different concentrations was given orally to the mice 24 hrs before and 2, 24, 48 and 72 hrs after virus inoculation. [a]Scores 0-4 assigned to % visible consolidation. *The difference from virus control is not significant ($P < 0.05$).

binations proved to be more efficient than the single agents. There was an additional reduction of mortality rates, virus lung titers, and pathomorphological changes in the lungs; the calculated combined effect ranged from additive to synergistic. The drug combinations were well tolerated by the experimental animals and the improved protection was not associated with an increased toxicity.

Discussion

Intranasal inoculation of influenza A/Aichi (H3N2) virus to mice produced a damaging infection of the lungs which, depending on the dose of the viral inoculum, was highly lethal to the animals. Our results showed that CM had a weak protective effect in the lethal infection, related obviously to its immunostimulating and immunorestaurating activities as no selective virus-inhibitory effect was found. In a sublethal influenza infection, CM did not reduce significantly the virus infectious titers in mouse lungs, determined on days 1, 3, 5, 7 p.i. Some activities of the immune cells determined in virus-infected and CM-treated mice at the same days p.i. revealed that CM caused corrections in the decreased functions of the immune cells. It has been found that CM induced a 5-fold increase of the number of Ma and a 2 – 6-fold increase of the migration ability of Ma in the healthy and infected animals, and did not cause significant changes in the phagocytic activity (Ivanova *et al.*, 1992). CM stimulated also the migration activity of polymorphonuclear lymphocytes (PMNs) and induced a 2-fold increase of the adherence ability of blood PMNs (Toshkova *et al.*, 1992). The pronounced stimulating effect of CM on the Ma and leukocyte functions could be due to the presence of a lipopolysaccharide (LPS) as proved by a mass-spectrometric (Gumpert *et al.*, 1982) and immunoelectron microscopic methods (Michailova *et al.*, 1984). This unusual localization of LPS was explained by the fact that the enzymes responsible for its synthesis in *Enterobacteriacea* are located in their cytoplasmic membranes (Osborn *et al.*, 1972). In the L-forms, lacking a cell wall, LPS remains associated with the cytoplasmic membrane. We presume that this particular situation resulted in a partial detoxication of LPS. In toxicological experiments run in parallel with LPS of *E. coli* O_{124} following the acute and chronic lethal toxicity, pyrogenicity and histopathological changes in kidneys, liver and heart, it has been shown that CM was 120-fold less toxic than the control LPS (Ivanova *et al.*, 1993), namely CM LD_{50} for mice was 120 mg/ml. Twenty-five mg/ml, previously determined as an optimal immunostimulating dose of CM (Ivanova, 1986) did not cause any toxic effects.

It has been shown previously that CM stimulated the functions of Ma in intact experimental animals as well as the proliferation of the antigen-binding T-lymphocytes, T-helper lymphocytes, B-lymphocytes and NK-cells (Ivanova, 1986), protected mice experimentally infected with *S. aureus*, *E. coli* and *K. pneumonia*, and showed a temporary immunorestaurative effect on the Ma and PMN functions of hamsters with myeloid virus-induced Graffi tumour (Ivanova *et al.*, 1991.

Phagocytes, consisting mainly of Ma and PMNs, were reported to be the crucial elements of resistance to an experimental infection in mice (Arora, 1993). As a non-specific immunostimulant, CM interferes with the functions of the cells of the immune system, Ma and PMNs (Ivanova *et al.*, 1992; Toshkova *et al.*, 1992), and due to its immunoprotective effects (Ivanova, 1986) it might elicit a state of resistance to the viral infection.

The immune response to experimental influenza infection is complex. Although some immune responses are induced or enhanced (NK-cell activity, IFN-α-production and the virus-specific T-lymphocyte response), other responses have been reported to be depressed (for review see Roberts and Domurat, 1989). The latter include depression of phagocytosis and killing of bacteria, depression of the lymphocyte proliferative response to phytohaemagglutinin (PHA) or other mitogens, and alteration of the chemotactic, oxidative and secretory functions of PMNs. These findings suggest that it would seem appropriate to use immunostimulatory substances alone or in combination to prevent or cure influenza virus infection.

Little is known concerning the effect of immunomodulators on influenza virus infection. Few substances with immunomodulatory activity were reported to be inhibitory to experimentally induced influenza virus infection – lentinan (Irinoda *et al.*, 1992), BCH-527 (Sidwell *et al.*, 1995) and MVE-2 (Carrano *et al.*, 1984). All these substances stimulated Ma functions, an effect which was also seen with CM (Ivanova *et al.*, 1992). Masihi *et al.* (1983) evaluated the effects of a mycobacterial preparation and muramyl dipeptide, Pancheva *et al.* (1990) found that isoprinosine potentiated the protective effect of rimantadine, and D'Agostini *et al.* (1996) reported the efficacy of combination therapy with amantadine, thymosin α1 and interferon α/ß.

In the present study, the combined treatment with the bacterial immunomodulator CM and rimantadine resulted in synergistic increase of protection, expressed by significant decrease of mortality and marked prolongation of survival times. Virus lung titers and lung consolidation in infected animals, treated with the combinations of the two substances were also reduced as compared to either single intervention. The combined use of CM and rimantadine did not lead to any enhancement either of acute or chronic toxicity.

The present results indicate a beneficial role of the combined use of a selective viral inhibitor and an immunostimulatory agent in the treatment of experimental influenza infection in mice. This could be explained by an effective integration of two different functions in the control of the infection. The specific antiviral agent inhibits the virus replication but does not affect the virus-induced immunosuppression, the immunomodulatory agent instead does not prevent the damage caused by the massive virus spread. Their combined application leads to effective interruption of the "vicious circle, established by viral infection (viral infection -> induction of immunosuppression -> enhanced spread of virus)" (Garaci *et al.*, 1994). We cannot predict that this conclusion could be of clinical importance for the influenza infection in humans. However, our results illustrate a potentially useful approach to its control.

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