Effect of Protein SV-IV on Experimental Salmonella enterica Serovar Typhimurium Infection in Mice

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Seminal vesicle protein IV (SV-IV) is a secretory anti-inflammatory, procoagulant, and immunomodulatory protein produced in large amounts by the seminal vesicle epithelium of the rat under the strict transcriptional control of androgen. In particular, this protein was shown to possess the ability to markedly inhibit in vivo the humoral and cell-mediated immune responses of mice to nonbacterial cellular antigens (sheep erythrocytes and spermatozoa). We report data that demonstrate that in mice treated with SV-IV and infected with Salmonella enterica serovar Typhimurium, SV-IV is able to downregulate some important immunological and biochemical parameters that serovar Typhimurium normally upregulates in these animals. This event did not correlate with a lower bacterial burden but was associated with a markedly increased one (300%). Furthermore, the treatment of mice with SV-IV alone also produced a significant increase in the rate of mortality among serovar Typhimurium-infected animals. The mechanism underlying these phenomena was investigated, and the strong immunosuppression produced by SV-IV in serovar Typhimurium-infected mice was suggested to be the basis for the increased rate of mortality. The SV-IV-mediated immunosuppression was characterized by a decrease in the humoral and cell-mediated immune responses, altered lymphocyte-macrophage interaction, downregulation of cytokine and inducible nitric oxide synthase gene expression, inhibition of macrophage phagocytosis and intracellular killing activities, and absence of apoptosis in the splenocyte population of SV-IV- and serovar Typhimurium-treated mice. The immunosuppressive activity of SV-IV was specific and was not due to aspecific cytotoxic effects. SV-IV-specific receptors (Kd = 10⁻⁸ M) occurring on the macrophage and lymphocyte plasma membranes may be involved in the molecular mechanism underlying the SV-IV-mediated immunosuppression. Some results obtained by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay also revealed a functional impairment of mitochondria (a decrease in mitochondrial dehydrogenase activity), thus indicating the possible implication of these organelles in the immunosuppressive process.

Seminal vesicle (SV) protein 4 (SV-IV; according to its mobility by sodium dodecyl sulfate [SDS]-polyacrylamide gel electrophoresis [PAGE]) is a small (Mr, 9,758), basic (pI 8.9), thermostable, secretory protein produced in large amounts by the SV epithelium of the adult rat under the strict transcriptional control of androgen (1, 15, 24). Proteins immunologically related to SV-IV have also been identified in several rat tissues (uterus, lung, liver, brain, etc.) as well as in human seminal fluid and SV secretions (2, 17, 18). The SV-IV protein has been purified to homogeneity and extensively characterized (1, 2, 15, 17, 18, 24, 25, 35). Its 90-aa (90 amino-acid) sequence is known, and the gene coding for it has been isolated, sequenced, and expressed in Escherichia coli (6, 12, 13, 16). SV-IV is a very flexible molecule in that aqueous solution behaves as a concentration-dependent self-associating system in which the degree of association (monomer ⇄ dimer ⇄ trimer equilibrium) appears to control the biological properties of the protein (35). The biological function of SV-IV is multifaceted. SV-IV is a remarkable bioactive protein due to its powerful non-species-specific anti-inflammatory, procoagulant, and immunomodulatory properties (3, 7, 8, 9, 11, 14, 19, 21, 28, 31, 37, 39). The anti-inflammatory activity of SV-IV is related to its ability to inhibit phospholipase A₂, the first enzyme of the arachidonate cascade (3, 19), while its procoagulant activity has been ascribed to its ability to inhibit antithrombin III (7–9). The modulatory effects of SV-IV on the humoral and cell-mediated immune responses are produced by its interference with macrophage-T cell cooperation (modulation of cytokine release and biological activity, inhibition of the macrophage antigen presentation activity, inhibition of the T-lymphocyte activation process) (19, 28, 31, 37). When it is transformed into a complex polymer by glutaminase (EC 2.3.2.13), the protein also has the ability to bind to the surfaces of epididymal spermatozoa, markedly decreasing their strong immunogenicity. It has been suggested that this biochemical event makes a critical contribution to immunoprotection of the spermatozoa during its perilous journey toward the egg in the immunologically competent female genital tract (20, 26, 28–30). The protein has also been found to possess a potent activating effect on the horseradish peroxidase and the selenium-dependent glutathione peroxidase (V. Metafora, F. Morelli, and S. Metafora, unpublished results), enzymes that are known to play important roles in the physiological mainte-
nance of cell redox equilibrium. Furthermore, we have recently found that SV-IV has a marked ability to inhibit the apoptosis induced in vitro in Raji cells by serum withdrawal (Metafora et al., unpublished results). Another interesting biochemical property of SV-IV is defined by its ability to promote a lymphocyte cytotoxic activity against the lymphoblastoid Raji cell line in human peripheral blood mononuclear cells (PBMCs) (27). We have experimental evidence that the cytotoxic effectors of this activity are functionally activated natural killer cells (27).

On the basis of these data and considerations, experiments were planned to verify whether the SV-IV protein is able to exert its immunomodulatory activity in mice infected with sublethal doses of pathogenic microorganisms (Salmonella enterica serovar Typhimurium).

**Materials and Methods**

Mice. BALB/c male mice (weight, 20 to 25 g; diet, 70K Mignini [Petrigiani, Perugia, Italy]) were maintained in a temperature-controlled animal house (20 ± 2°C) with automatic 12-h lighting cycles.

Microorganism. The microorganism used was S. enterica serovar Typhimurium NCTC 74 grown in nutrient broth (Difco Laboratories, Detroit, Mich.).

Purification of protein SV-IV. Milligram amounts (400 mg) of protein SV-IV were purified to homogeneity from SV-IV secretions of adult Fisher-Wistar rats by a published procedure by using ion-exchange and gel filtration column chromatography (15). The purity of the protein was evaluated by SDS-PAGE under a published procedure by using ion-exchange and gel filtration column chromatography (15). The purity of the protein was evaluated by SDS-PAGE under denaturing and nondenaturing conditions (17), amino acid composition analysis, the fingerprinting technique (1), and fast atom bombardment mass spectrometry (30). The SV-IV preparations were completely free of lipopolysaccharide (LPS) (30).

The purified protein was characterized by its ability to promote a lymphocyte cytotoxic activity against the lymphoblastoid Raji cell line in human peripheral blood mononuclear cells (PBMCs) (27). We have experimental evidence that the cytotoxic effectors of this activity are functionally activated natural killer cells (27).

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Evaluation of anti-serovar Typhimurium antibody titer. A conventional agglutination test (10) was used to evaluate the anti-serovar Typhimurium antibody titer in sera obtained from groups (groups A, C, and D) of mice treated by the standard protocol described above. Briefly, serum samples serially diluted two-fold in PBS were prepared in final volumes of 0.1 ml in glass test tubes (10 by 75 mm). A serovar Typhimurium suspension (0.05 ml; optical density at 550 nm, 0.600/ml) was added to each tube, the contents of the tubes were mixed, the tubes were covered with Parafilm and incubated at 37°C and the results were read after 24 h. The FACS analysis demonstrated that the immunoglobulins occurring in the analyzed antisera were mainly immunoglobulin G (IgG). Similar anti-serovar Typhimurium antibody titers for comparison were obtained by calculating these titers either as an average of the various titers obtained for the single animals belonging to each group or as the titers for the pooled sera obtained from the different groups of mice.

FACS analysis. Spleenocytes obtained from groups of mice treated by the standard protocol (groups A, C, and D) were stained for immunofluorescence analysis by an indirect labeling procedure. Unconjugated antibodies (anti-CD3 and anti-surface IgG [anti-sIgG] from Becton Dickenson [Mountain View, Calif.]; anti-1a and anti-Mac1 from Serotec) were used as the first staining reagents, whereas fluorescein isothiocyanate-labeled xenogeneic polyspecific or IgG class-specific antibodies were used as the second staining reagents. Control indirect staining procedures that included only the second staining reagent were routinely carried out. Following immunostaining, the cells were counterstained with propidium iodide to allow exclusion of dead cells (brightly stained with propidium iodide) from the immunofluorescence analysis. In particular, before testing for Ia the CD3+ cells were separated from other spleenocytes by affinity chromatography on an R & D Systems column. Cell fluorescence was analyzed with a cytofluorograph (FACScan; Becton Dickinson, Mountain View, Calif.) by using the 488-nm emission line of an argon laser. Macrophages were identified and separately analyzed on the basis of their characteristic combination of forward, right-angle scatter and their positive fluorescence staining with the specific monoclonal antibodies. The percentage of fluorescence-positive live cells, the mean fluorescence, and the scatter values of fluorescence-positive cells were determined with a Hewlett-Packard computer system. In sandwich staining procedures, the percentage of specifically stained cells was determined by subtracting the percentage of positive cells present in the stained control (the layer stained only with the second staining reagents) from that obtained after staining with both the first and the second reagent antibodies.

Mitogen-induced T- and B-cell proliferation. One hundred microliters of spleenocyte suspensions (3 × 10^6 cells) prepared from groups of mice treated by the standard protocol (groups A, C, and D) and was added to the wells of 96-well, round-bottomed microtiter plates containing 100 μl of mitogen solution (24 μg of concanavalin A [ConA; Sigma, Milan, Italy] per ml or 20 μg of LPS from E. coli O128:B12 [Sigma per ml] in complete RPMI 1640 medium. The plates were incubated at 37°C in an incubator with a humidified atmosphere consisting of 5% CO2 and 95% air for 72 h. Six hours before the administration of which was administered intraperitoneally (i.p.) on days 1, 7, and 14 of the PBS treatment (group C), respective mice were injected with SV-IV alone (group D) or PBS alone. Mice that received SV-IV in PBS (5 nmol/mouse) for 14 days was infected with three sublethal doses (4 × 10^5 CFU/mouse) of serovar Typhimurium, each of which was administered i.p. on days 1, 7, and 14 of the SV-IV treatment (group D), respectively. Blood, spleenocytes, and peritoneal macrophages obtained from the four groups of animals (groups A, B, C, and D) at different times (3, 7, 12, and 21 days) after the end of the 14 days of treatment with PBS alone, SV-IV alone, PBS and serovar Typhimurium, or SV-IV and serovar Typhimurium were used to perform the majority of the experiments whose results are reported in the Results section.

In the alternative protocol, groups of mice belonging to group A or B were inoculated i.p. with either PBS (control) or a 50% lethal dose (LD_{50}) dose (8 × 10^7 CFU/mouse) of serovar Typhimurium on day 3, 7, 14, or 21 after the end of the PBS or the PBS and SV-IV treatment. Forty-eight hours after the i.p. inoculation the percent mortality and intracellular bacterial clearance abilities of control or serovar Typhimurium-infected animals were evaluated by the procedures described below.

In preliminary experiments we have found that the optimal in vivo immunosuppressive effect of SV-IV in our murine model was detectable in the range of 2 to 8 nmol of SV-IV/mouse. On this basis, we decided to use in all the experiments reported in this paper an SV-IV concentration of 0.1 μmol/ml. High or lower concentrations of SV-IV always produced significantly lower immunosuppressive effects, with the decrease in the biological effect of SV-IV at higher concentrations probably being related to the self-associative concentration-dependent properties of this protein in aqueous solutions (formation of biologically inactive SV-IV trimers).

The microorganism used was S. enterica serovar Typhimurium NCTC 74 grown in nutrient broth (Difco Laboratories, Detroit, Mich.).

Purification of protein SV-IV. Milligram amounts (400 mg) of protein SV-IV were purified to homogeneity from SV-IV secretions of adult Fisher-Wistar rats by a published procedure by using ion-exchange and gel filtration column chromatography (15). The purity of the protein was evaluated by SDS-PAGE under denaturing and nondenaturing conditions (17), amino acid composition analysis, the fingerprinting technique (1), and fast atom bombardment mass spectrometry (30). The SV-IV preparations were completely free of lipopolysaccharide (LPS) (30).

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harvesting of the cells. 0.5 μCi of [methyl-3H]thymidine (5 Ci/mmol; Amersham, Little Chalfont, United Kingdom) was added to each well and thymidine was continued for another 6 h. All cultures were harvested with a 12-well automated cell harvester and placed onto glass fiber filters. The filters were dried, and their radioactivities were measured with a Beckman liquid scintillation counter. All the determinations were carried out in triplicate.

Evaluation of animal mortality and enumeration of numbers of Salmonella CFU in serovar Typhimurium-infected mice. The effect of SV-IV treatment (5 nmol/mouse for 14 days) on the mortality of S. enterica serovar Typhimurium-infected mice was evaluated in animals inoculated i.p. with 8 × 107 CFU serovar Typhimurium cells (LD50) in PBS at different times (3, 7, 15, and 21 days) after the end of SV-IV treatment (alternative protocol). The mean mortality rates for control mice (PBS-treated mice; group A) and experimental mice (SV-IV-treated mice; group B) were recorded 48 h after infection, and the data are expressed in percent.

Groups of three serovar Typhimurium-infected mice that survived infection with the LD50 were killed by cervical dislocation, and their spleens and livers were aseptically removed and homogenized separately in 2 ml of cold PBS with a sterile glass-Teflon Potter-Elvehjem motor-driven homogenizer. Serial dilutions of the homogenates in sterile PBS were plated on nutrient agar. The numbers of CFU were carefully counted after overnight incubation at 37°C, and the counting results were expressed as the log10 number of bacteria (CFU) per organ.

Phagocytosis assay and killing index determination. The phagocytic activities of the peritoneal macrophages obtained from mice treated by the standard protocol were expressed by the phagocytic index determined with suspended cells by a previously published procedure (38). Briefly, the cells, suspended in complete RPMI 1640 medium (2 × 105/ml), were incubated at 37°C with Staphylococcus epidermidis (107 cells/ml) as target cells. Following 60 min of incubation, 0.5 ml of the sample was added to 1.5 ml of ice-cold complete RPMI 1640 medium to stop the phagocytosis, and the sample was centrifuged at 110 × g for 4 min. Two aliquots (0.1 ml) of three consecutive dilutions of the supernatant were plated. Plates with 10 to 500 colonies were counted, the number of bacteria in the supernatant was calculated, and the phagocytic index was determined.

After 60 min of incubation at 37°C to allow phagocytosis, the noningested bacteria were separated from the macrophages by centrifugation (at 110 × g for 4 min) and the macrophages were washed twice at 4°C with ice-cold PBS. The sedimented macrophages were lysed by freezing and thawing, and the numbers of viable intracellular bacteria in the lysates were determined. The level of intracellular killing at 60 min was expressed as the number of viable intracellular bacteria (killing index) by the formula log N0 – log Nf, in which N0 is the number of viable intracellular bacteria at time zero and Nf is the number of viable intracellular bacteria at 60 min. Controls were prepared with macrophages not incubated with Staphylococcus to account for the possible occurrence of viable S. enterica serovar Typhimurium or other contaminating bacteria in the different lysates analyzed.

DNA fragmentation analysis: electrophoresis and TUNEL. In order to evaluate by agarose gel electrophoresis internucleosomal DNA fragmentation, a typical fragmentation event was evaluated. Mice infected with serovar Typhimurium, mice treated with PBS alone, SV-IV in PBS alone, or PBS-serovar Typhimurium, or SV-IV-serovar Typhimurium by the standard protocol were reverse transcribed with random nanomers and Superscript II reverse transcriptase ( Gibco-BRL) at 37°C for 1 h. The reaction was terminated by heating the reverse transcriptase incubation mixture at 95°C for 5 min, followed by quick chilling on ice. Negative controls for the amplification reactions were obtained by performing RNA transcription in the absence of reverse transcriptase. Two microsomes of the synthesized cDNA was amplified using a 30-cycle reaction, and the PCR products were separated on a 2% agarose gel (2% Tris-HCl (pH 8.3), 1.5 mM MgCl2, 50 mM KCl, 200 μM concentration of each of the deoxyribonucleoside triphosphates, 0.5 U of Taq DNA polymerase Gold (Perkin-Elmer), and 100 ng of both sense and antisense murine primers for gamma interferon (IFN-γ; sense primer, 5′-TGCATCCTGGTCTTGACGTC TTCCCTCATGCG-3′; antisense primer, 5′-TGGACCTGGTGTGGTGTACCC TCAACTTGCGC-3′), interleukin-10 (IL-10; sense primer, 5′-CTGGAAAGCC AAGGTTGTCTAC-3′; antisense primer, 5′-GAGCTGTCGAGAAGATGTTA G-3′), IL-5 (sense primer, 5′-GACAAGCAATTAGACAGATGAGG-3′; antisense primer, 5′-CCGTGATACCTCAGAGAATGC-3′), IL-4 (sense primer, 5′-ATGGGTCTCAACCTGCAAGAGT-3′; antisense primer, 5′-GAGCTGTCGAGAAGATGTTAG-3′), IL-3 (sense primer, 5′-GACAAGCAATTAGACAGATGAGG-3′; antisense primer, 5′-CGCGATACCTCAGAGAATGC-3′), TNF alpha (TNF-α; sense primer, 5′-TCCTGCTACTCAAGCTTGCGT-3′; antisense primer, 5′-GATGATAGATGAACTCGGCGTT-3′), and nitric oxide synthase (iNOS; sense primer, 5′-CTCGGTTACCTCAGAGAATGC-3′; antisense primer, 5′-ATGGGTCTCAACCTGCAAGAGT-3′).

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contrast, as internal controls for the IFN-γ and TNF-α analyses, sense and antisense primers for the mouse β-actin gene were added to the PCR assay mixtures. The PCR products were synthesized by using the HPRT-specific primers (sense primer, 5’-CCTGATTAGTCGCAATATCCAGG-3’; antisense primer, 5’-GACTACCTCATGAAGATCCT-3’; antisense primer, 5’-GCTGTGATCCACATCTGC-3’; antisense primer, 5’-GACTACCTCATGAAGATCCT-3’; antisense primer, 5’-GCTGATCCACATCTGC-3’; antisense primer, 5’-GACTACCTCATGAAGATCCT-3’). The PCR products were performed in a DNA thermal cycler (GeneAmp PCR system 9700; Perkin-Elmer-Applied Biosystems). The HPRT reverse transcription-PCR (RT-PCR) coamplification protocol for piglets was followed. The PCR products were added to the samples for the amplification reaction mixture 8 cycles later than the time of addition of the other interleukin- or iNOS-specific primers. The identities of these products were confirmed by the “primer-dropping” method (40), so that the amplification remained in the exponential phase. The PCR products were analyzed by electrophoresis on an ethidium bromide-containing 1.2% agarose gel in TBE. The identities of these products were confirmed by comparing their sizes with the size expected from the known gene sequence and by direct nucleotide DNA sequencing. Quantification of each PCR product was achieved by integrating the peak area in the agarose gel. The values in parentheses are the number of dead animals/total number of animals.

RESULTS

SV-IV pretreatment of S. enterica serovar Typhimurium-infected mice increases the animal mortality rate and produces a marked decrease in intracellular bacterial clearance in spleen and liver macrophages. The effect of SV-IV (5 nmol/mouse for 14 days) on the mortality rate and the in vivo intracellular bacterial clearance for mice injected with a single LD_{50} of serovar Typhimurium (8 × 10^9 cells) at different times (3, 7, 15, or 21 days) after the end of SV-IV treatment by the alternative treatment protocol (see Materials and Methods) was evaluated, and the relevant data are reported in Tables 1 and 2, respectively. At this point, it is noteworthy that as a consequence of the large doses of serovar Typhimurium used in this protocol, the death of the infected animals was rapid (few days) and was caused by a severe endotoxic shock. From these data it appears that the percent mortality of the infected mice progressively increased with time, reaching a maximum of 85% 21 days after the end of SV-IV treatment (Table 1). Concurrent with the increase in the animal mortality rate, a marked increase (about 65%) in the number of intracellular living bacteria in serovar Typhimurium-infected spleen and liver macrophages was already detectable by day 3 after the end of SV-IV treatment. The bacterial burden in these cells increased progressively with time, reaching a maximum (about 300%) 21 days after the SV-IV treatment. It is interesting that the increase in the organism burden was about the same (300%) when the experiment was repeated with the standard treatment protocol.

SV-IV inhibits the mouse humoral immune response to serovar Typhimurium infection. To investigate the mechanism of the increase in the mortality rate induced by SV-IV in mice infected with the LD_{50} of serovar Typhimurium, we used another experimental protocol, the standard protocol (see Materials and Methods), in which SV-IV or PBS was subcutaneously injected every day for 14 days, whereas the serovar Typhimurium infection was obtained by i.p. inoculation of three separate sublethal doses of serovar Typhimurium, each given at 7-day intervals over the same total period of time. The rationale behind this experimental design was mainly related to the requirement of three separate serovar Typhimurium inoculations in a total time of 14 days to elicit an optimal anti-

| Mouse treatment | Mortality (%) on the following days for mice inoculated with the LD_{50} of S. enterica serovar Typhimurium |
|----------------|---------------------------------------------------------------------|
| PBS (control)  | 50 ± 4 (25/50) 50 ± 3 (25/50) 50 ± 6 (25/50) 50 ± 5 (25/50) |
| SV-IV (5 nmol/mouse) | 68 ± 2 (34/50) 76 ± 6 (38/50) 80 ± 4 (40/50) 86 ± 7 (43/50) |

* The mice were treated by the alternative protocol. Experimental details are given in Materials and Methods. The data represent the means ± SEMs of six independent determinations, in which each point was determined from assays performed in triplicate. The number of days refers to the time of inoculation of the mice with the LD_{50} of serovar Typhimurium following their treatment with SV-IV or PBS. Further experimental details are given in Materials and Methods.

### Statistical analysis

The data reported as means ± SEMs obtained from six independent determinations, in which each point was determined from assays performed in triplicate. The means were compared by analysis of variance plus Bonferroni’s t test, and a P value of less than 0.05 was considered significant.

### RESULTS

**SV-IV treatment Organ**

| Bacterial count (log_{10} CFU/organ) | Mouse treatment | Organ |
|-------------------------------------|-----------------|-------|
|                                    | PBS (control)   | Spleen 0.6 ± 1.3 8.6 ± 1.6 7.5 ± 1.7 8.2 ± 1.4 |
|                                    | Liver 7.2 ± 1.5 9.0 ± 1.4 6.5 ± 1.0 7.4 ± 1.3 |
|                                    | SV-IV (5 nmol/mouse) Spleen 10.8 ± 3.0 16 ± 4.8 25.7 ± 4.5 34 ± 5.5 |
|                                    | Liver 11.3 ± 3.3 18 ± 5.0 26 ± 5.3 32 ± 5.0 |

* The mice were treated by the alternative protocol. Bayer experimental details are given in Materials and Methods. The data represent the means ± SEMs of six independent determinations, in which each point was determined from assays performed in triplicate. The number of days refers to the time of inoculation of the LD_{50} of serovar Typhimurium after treatment of the mice with SV-IV or PBS.

**P < 0.01 (by Bonferroni’s t test) versus the control value.**
TABLE 3. Total numbers of splenocytes per spleen and their main cell subsets in mice killed 7 days after the end of the standard treatment*

| Animal treatment          | No. of splenocytes/spleen (10^6) | No. of cells (10^6) of the following splenocyte subset: |
|---------------------------|-----------------------------------|--------------------------------------------------------|
|                           |                                   | CD3^+ | CD3^+/Ia^+ | slgG^+ | Mac1^+ |
| PBS (control)             | 85 ± 4                            | 34 ± 3 | 1.0 ± 0.4  | 42 ± 5  | 10 ± 2  |
| PBS-serovar Typhimurium   | 165 ± 15                         | 63 ± 5 | 18 ± 4.0^a | 68 ± 7^b | 35 ± 6^c |
| SV-IV-serovar Typhimurium | 82 ± 5                           | 26 ± 2 | 1.2 ± 0.3  | 36 ± 3  | 17 ± 3  |

*The mice were treated by the standard protocol. Experimental details are given in Materials and Methods. Each value represents the mean ±SEM of six independent determinations, in which each point was determined from assays performed in triplicate. CD3^+, T lymphocytes; CD3^+/Ia^+, Ia^+ T lymphocytes; slgG^+, B lymphocytes; Mac1^+, macrophages.

a P < 0.01 (by Bonferroni’s t test) versus the control value

The immunologic reactivities of splenocytes isolated from serovar Typhimurium-infected mice were measured by evaluating their abilities to respond in vitro to the mitogen LPS or ConA at different times (3, 7, 14, and 21 days) after the end of the standard treatment with serovar Typhimurium and SV-IV. The data in Table 4 demonstrate that SV-IV did not block the responses to the mitogen LPS or ConA, but it blocked some of the increase induced by serovar Typhimurium, keeping the values closer to control levels. Consistent with these results are some previously published data (31) that also showed that treatment of mice with SV-IV alone is able to reduce the humoral and cell-mediated immune responses of these animals to nonbacterial cellular antigens (sheep erythrocytes and spermatozoa) and is able to keep the values of the parameters studied close to control levels.

The cytokine mRNA patterns of splenic lymphocytes and macrophages obtained from mice treated with the standard protocol define the in vivo immunosuppressive properties of SV-IV. Groups of mice were treated with PBS alone, SV-IV and PBS, PBS and serovar Typhimurium, or SV-IV and serovar Typhimurium by the standard protocol. Three days (early phase) and 7 days (late phase) after the end of the experimental treatment, the splenocytes obtained from treated or untreated (control) mice were separated into adherent macrophages and nonadherent lymphocytes, and the levels of a variety of cytokine mRNAs (IFN-γ, IL-10, IL-4, and IL-5 mRNAs in lymphocytes and TGF-β, TNF-α, and IL-1α mRNAs in macrophages) present in their total RNA was monitored by semiquantitative RT-PCR. The data reported in Fig. 2A to D indicate that the splenic lymphocyte cytokine mRNA profile in the early phase after the end of the PBS-serovar Typhimurium treatment reflects a Th1 and Th2 cytokine expression pattern, characterized by the concurrent production of IFN-γ, IL-4, IL-5, and IL-10; in the late phase, the cytokine pattern reflects, instead, a predominantly Th1 pattern, characterized by the presence of IFN-γ and IL-10 and the absence of IL-4 and IL-5. In contrast, when the animals were treated with either SV-IV–PBS or SV-IV–serovar Typhimurium, the transcriptional activities of the splenic lymphocyte cytokine genes under study were dramatically reduced (particularly evident in the late phase) as a consequence of the SV-IV immunosuppressive properties (Fig. 2A to D). The latter result was confirmed by splenic macrophage cytokine gene expression analysis, which indicated a marked downregulation of the mRNAs coding for TGF-β, TNF-α, and IL-1α (Fig. 2E, F, and G, respectively; the data for the early phase [not shown in Fig. 2] exactly overlapped the data for the late phase).

Release of IL-1α and IL-1β from LPS-stimulated peritoneal macrophages is markedly inhibited by SV-IV in vitro. In vitro experiments were carried out to confirm in our murine model the known capacity of SV-IV to inhibit the release of cytokines (in this case, IL-1α and IL-1β) from human macrophages stimulated with LPS (39), a major component of the serovar Typhimurium outer membrane. The data reported in Table 5 demonstrate that SV-IV has a marked inhibitory effect on the release of both IL-1α (about 50%) and IL-1β (about 85%) into the murine LPS-stimulated macrophage culture medium. This finding was probably related to the SV-IV-mediated down-regulation of IL-1α synthesis at the transcriptional level (Fig. 2G). The low levels of IL-1β released from LPS-stimulated macrophages probably reflect the lower level of IL-1β production relative to the level of IL-1α production typical of mice.

Treatment of serovar Typhimurium-infected mice with SV-IV markedly decreases the phagocytic and intracellular killing indices of their peritoneal macrophages. The in vitro phagocytic and intracellular killing activities of peritoneal macrophages obtained from mice treated with PBS alone, PBS-serovar Typhimurium, or SV-IV–serovar Typhimurium by the
FIG. 1. (A) Suppressive effect of SV-IV on the mouse humoral immune response to sublethal serovar Typhimurium infection (standard protocol). (B) Nitric oxide production by splenic macrophages (10^7) obtained from mice injected with PBS or SV-IV for 14 days and concurrently infected with sublethal doses of serovar Typhimurium by the standard protocol. (C) Mitochondrial dehydrogenase activity (MTT test) of splenocytes obtained from animals infected with sublethal doses of serovar Typhimurium in the presence or absence of SV-IV (standard protocol). The extent of MTT reduction to formazan within the living cells, which is proportional to the mitochondrial dehydrogenase activity, was quantitated by measuring the optical density at 550 nm of the solubilized material with a Molecular Devices microplate reader. Open bars, mice injected with PBS only (control); bars with diagonal stripes, serovar Typhimurium-infected mice injected with PBS only; solid bars, serovar Typhimurium-infected mice injected with SV-IV. Experimental details are reported in Materials and Methods. The data represent the means ± SEMs of triplicate determinations. *, P < 0.05 versus the serovar Typhimurium-infected mice injected with PBS; **, P < 0.01 versus the serovar Typhimurium-infected mice injected with PBS.
The mice were treated by the standard protocol. Experimental details are described in Materials and Methods. Each value represents the mean ± SEM of three separate experiments, in which each point was determined from assays performed in triplicate. These data are highly significant, because their P values are 0.01 (by Bonferroni's test) compared with the control value.

Table 4. In vitro response to LPS or ConA of splenocytes of mice treated with PBS alone (group A), 0.5% Serovar Typhimurium alone (group C), or the SV-IV and Serovar Typhimurium combination (group D) at different times after the end of the standard treatment. The data reported in Table 6 indicate that in the SV-IV-treated mice both in vitro phagocytic and intracellular killing activities decreased progressively with time, reaching a minimum of about 73% when the animals were killed 21 days after the last SV-IV injection.

The SV-IV treatment of serovar Typhimurium-infected mice decreases markedly the ability of their peritoneal macrophages to produce NO and iNOS mRNA. The data reported in Fig. 1B show that the ability of the peritoneal macrophages from mice treated with SV-IV and serovar Typhimurium by the standard protocol to produce NO was markedly inhibited in comparison with that of peritoneal macrophages from control mice treated with PBS and serovar Typhimurium. The inhibition increased with time, reaching the maximum (about 90%) 21 days after the SV-IV and serovar Typhimurium treatment. To verify whether the SV-IV-mediated inhibition of NO production was related to a downregulation of iNOS gene expression, we prepared cDNA from the total RNA of splenic macrophages obtained from mice treated with PBS alone (control), SV-IV in PBS, SV-IV and serovar Typhimurium, or PBS and serovar Typhimurium. The amount of iNOS mRNA in this cDNA was evaluated by semiquantitative RT-PCR, which demonstrated that iNOS gene expression, which was undetectable in control animals, was significantly stimulated in serovar Typhimurium-infected mice (Fig. 2H, lane STM [serovar Typhimurium]) 7 days after the end of the experimental treatment. The SV-IV treatment of the latter animals produced a marked downregulation of iNOS gene transcriptional activity (Fig. 2H, lane STM [serovar Typhimurium] + SV4 [SV-IV]). Similar results were obtained 21 days after the end of the experimental treatment (data not shown).

Mitochondrial damage not associated with classic apoptotic signs is detectable in the splenocytes of mice treated with SV-IV and serovar Typhimurium. In the previous paragraphs we have reported data that strongly suggest the possibility that an SV-IV-mediated immunosuppressive mechanism could be the basis for the marked increase in the rate of mortality that occurs in mice treated with the combination of SV-IV and serovar Typhimurium. To explore the involvement of apoptosis in this mechanism, we evaluated by TUNEL analysis and a DNA fragmentation electrophoretic assay the occurrence of apoptotic death in the splenocyte population obtained from mice treated with either PBS alone, SV-IV in PBS, PBS and serovar Typhimurium, or SV-IV and serovar Typhimurium by the standard protocol. The results obtained demonstrated that the number of apoptotic events in all splenocyte populations analyzed (data not shown) was not significant, whereas in the apoptosis-positive control evaluation performed with splenocytes treated with 250 µM H$_2$O$_2$ at 37°C for 6 h, more than 50% of the cell population was clearly apoptotic (data not shown).

In contrast to these findings, the data from the MTT assay reported in Fig. 1C show that a progressive decrease with time of the mitochondrial dehydrogenase activity (expression of a functional mitochondrial damage) in splenocytes of mice treated with SV-IV and serovar Typhimurium was especially evident (about 70%) 21 days after the end of the standard experimental treatment. It is noteworthy that a similar but smaller reduction of the activity of the same enzyme (about
FIG. 2. Semiquantitative RT-PCR analysis of cytokine (A to G) or iNOS (H) mRNA expression in lymphocytes (A to D) or macrophages (E to H) obtained from the spleens of mice 3 and 7 days after the end of the standard treatment with SV-IV and serovar Typhimurium, alone or in combination. Total RNA was isolated from $10^7$ splenocytes obtained from untreated mice (control mice injected with PBS only) or treated mice (serovar Typhimurium-infected mice injected with PBS [STM]; mice injected with PBS and SV-IV [SV4]; serovar Typhimurium-infected mice injected with SV-IV [STM + SV4]) and amplified by RT-PCR with specific primer pairs. Experimental details are reported in Materials and Methods. No products were detectable in control amplifications performed in the absence of cDNA (negative control; control (--)). The semiquantitative evaluation of each PCR product was achieved by integrating the peak area obtained by densitometry of the ethidium bromide-stained agarose gels (with NIH image software, version 16). The ratio between the yield of each amplified product and that of the coamplified internal control allows a relative estimate of mRNAs levels in the samples analyzed. The ratios were as follows, from left to right: IFN-γ/β-actin mRNA ratio, 3.22, 3.16, 2.65, 0.20, 1.64, 1.00, 0.63, and 0.005; IL-10/HPRT mRNA ratio, 0.17, 6.71, 4.47, 3.15, 4.26, 4.28, 3.35, and 1.02; IL-5/HPRT mRNA ratio, 0.08, 1.04, 0.10, 0.13, 0.15, 0.06, and 0.05; IL-4/HPRT mRNA ratio, 0.13, 1.03, 0.12, 0.10, 0.10, 0.07, 0.06, and 0.06; TGF-β/HPRT mRNA ratio, 0.03, 1.98, 0.50, and 0.02; TNF-α/β-actin mRNA ratio, 0.59, 1.9, 1.29, and 1.54; IL-1α/HPRT mRNA ratio, 0.08, 2.33, 0.08, and 0.28; iNOS/HPRT mRNA ratio, 0.01, 1.67, 0.02, and 0.01.
TABLE 5. Effect of SV-IV on LPS-mediated induction of IL-1 release from mouse peritoneal macrophages

| Addition to culture | Extracellular IL-1 concn (pg/ml) |
|---------------------|-------------------------------|
|                     | IL-1α                        | IL-1β                        |
| None (control)      | 122 ± 15                     | 45 ± 12                      |
| LPS (20 µg/ml)      | 8,750 ± 754<sup>a</sup>      | 1,055 ± 43<sup>b</sup>      |
| SV-IV (15 µM)       | 135 ± 20                     | 54 ± 7                       |
| LPS (20 µg/ml) + SV-IV (15 µM) | 4,440 ± 636<sup>b</sup> | 152 ± 25                     |

<sup>a</sup> Murine peritoneal macrophages were cultured in RPMI 1640 complete medium in the presence or absence of LPS, with or without SV-IV. The IL-1α and IL-1β released into the culture medium were quantified as described in Materials and Methods. Each value represents the mean ± SEM of three separate experiments, in each of which each point was determined from assays performed in triplicate.

<sup>b</sup> P < 0.01 (by Bonferroni’s t test) versus the control value.

25%) was also detectable in splenocytes prepared from mice treated with serovar Typhimurium alone 21 days after the last serovar Typhimurium inoculation (Fig. 1C).

DISCUSSION

The infection of SV-IV-treated mice with a single LD₅₀ of S. enterica serovar Typhimurium on day 3, 7, 15, or 21 after the end of the SV-IV treatment (alternative protocol) produced a significant increase in the rate of mortality among these animals that was associated with a marked reduction of the intracellular bacterial clearance abilities of their spleen and liver macrophages. The SV-IV treatment of mice concurrently infected with sublethal doses of serovar Typhimurium (standard protocol) produced a decrease in the in vitro phagocytosis and intracellular killing activities of their peritoneal macrophages. The reduction in the level of clearance of the bacteria from the spleens and livers and the decrease in the level of intracellular killing of the bacteria in the peritoneal macrophages of mice treated with SV-IV and serovar Typhimurium were probably related to a marked downregulation in the abilities of these macrophages to produce NO (a free radical involved in the intracellular killing of bacteria) and iNOS. The inhibitory effect of SV-IV on iNOS gene expression could be related, in turn, to the binding of SV-IV to SV-IV–specific tyrosine kinase–associated receptors (Kₐ = 10⁻⁸ M; 80,000 to 100,000 SV-IV–specific binding sites/cell) (11, 19, 21, 35; Metafora et al., unpublished data), with the subsequent production of a protein phosphorylation cascade following the plasma membrane signal transduction process. In addition, as a result of the known ability of anti-inflammatory drugs to control the inflammatory process through the downregulation of the NF-kB gene expression pathway, the possible inhibition of NF-kB (41) by SV-IV (an anti-inflammatory protein) could reasonably be involved in the process. The SV-IV-mediated reduction of phagocytosis observed in serovar Typhimurium-infected mice confirms previous data from our laboratory that have demonstrated the in vitro inhibitory effects of SV-IV on human macrophage and polymorphonuclear leukocyte phagocytosis activities (11, 21).

The higher rate of mortality observed among serovar Typhimurium-infected mice treated with SV-IV in comparison with that observed among the PBS-treated animals could be related not only to the decrease in the level of macrophage phagocytosis and intracellular killing activity but also to the marked inhibition of the humoral and cell-mediated immune responses to the serovar Typhimurium infection. The mechanism of this SV-IV-mediated effect was investigated. The data reported in this paper show that in the spleens of mice treated with SV-IV and serovar Typhimurium a significant decrease in the total number of splenocytes and their main cell subsets (T and B cells, macrophages) was associated with a marked decrease in the total number of IA<sup>b</sup> splenic T cells and a strong inhibition of the ability of the splenocytes to respond in vitro to polyclonal mitogen stimuli (this may be the possible expression of SV-IV-mediated anergy). In addition, classic signs of apoptosis were not detected in these cells, although their mitochondria were found to be functionally damaged (MTT assay; see below). These findings may reasonably be related to a decrease in the level of cell proliferation resulting from both a marked reduction of the serovar Typhimurium-mediated macrophage and lymphocyte activation process and the establishment in these cells of a certain degree of SV-IV-mediated anergy instead of to an increase in the level of cell death. Taken together, these data strongly suggest that the inhibitory effect of SV-IV on the humoral immune response is the consequence of altered cooperation between macrophages and lymphocytes (inhibition of macrophage antigen presentation activity, inhibition of macrophage and lymphocyte cytokine synthesis and release, inhibition of lymphocyte activation and proliferation [19, 28, 31, 37]).

It is interesting that the in vivo immunosuppressive effects of SV-IV on serovar Typhimurium-infected mice correlate very well not only with the in vivo inhibitory action of SV-IV on the mouse humoral immune response to other cell antigens (sheep red blood cells and mouse epididymal spermatozoa) but also with the in vitro immunosuppressive action of SV-IV on LPS-, ConA-, or antigen-stimulated rat, mouse, or human PBMCs (19, 28, 31, 37).

The possibility that the immunosuppressive activity of SV-IV could be due to a specific cytotoxic properties of this protein

TABLE 6. Phagocytic and intracellular killing activities of peritoneal macrophages obtained from mice subjected to the standard protocol<sup>a</sup>

| Animal treatment | Phagocytic index | Killing index |
|------------------|-----------------|--------------|
| PBS-3-21         | 0.05 ± 0.01     | 0.10 ± 0.03  |
| PBS-serovar Typhimurium-3-21 | 0.61 ± 0.12 | 0.22 ± 0.05  |

<sup>a</sup> The mice were treated by the standard protocol. Experimental details are given in Materials and Methods. Each value represents the mean ± SEM of six independent determinations, in which each point was determined from assays performed in triplicate. PBS–3-21, peritoneal macrophages obtained at different times (3, 7, 14, or 21 days) after treatment with PBS alone; PBS–serovar Typhimurium–3-21, peritoneal macrophages obtained at different times (3, 7, 14, or 21 days) after treatment with PBS and S. enterica serovar Typhimurium; SV-IV–serovar Typhimurium, peritoneal macrophages obtained 3, 7, 14, or 21 days after treatment with PBS and S. enterica serovar Typhimurium.

<sup>b</sup> P < 0.01 (by Bonferroni’s t test) versus the control value.
protein can be ruled out by the following observations. (i) The immunosuppressive activity of SV-IV is detectable in vitro on activated PBMCs only at a relatively low range of concentrations (5 to 30 μM); higher concentrations become progressively ineffective (biphasic effect) as a consequence of the SV-IV concentration-dependent self-association equilibrium (at higher concentrations there is a shift from the active monomeric form to the biologically inactive, nontoxic dimeric or trimeric configuration) (35). (ii) The same protein at similar concentrations does not inhibit but, rather, stimulates in vitro the metabolic activities of these cells (19, 28). Such a discrepancy may be related to both the in vivo conditions and the particular strain of serovar Typhimurium (NCTC 1038/H9251/H9252). These findings suggest that mitochondria could play an important role (perhaps an involvement in the induction of anergy) in the molecular mechanism at the basis of the marked immunosuppression induced in mice by long-term (14 days) treatment with SV-IV. Experiments are in progress to verify this hypothesis.

The immunosuppressive effect of SV-IV in our murine model of experimental serovar Typhimurium infection can be related either to the direct inhibitory effect of SV-IV on the strong immunogenicity of some important components of the serovar Typhimurium outer membrane (LPS, porins, etc.) or to the negative interference of this protein with the signal transduction biochemical machinery of the macrophage and/or the lymphocyte plasma membrane. Against the first hypothesis there is the finding that SV-IV is unable to form in vitro a stable molecular complex with LPS (39). On the contrary, the second possibility is supported by the finding that SV-IV has the ability to interact with its target cells (lymphocytes, macrophages, polymorphonuclear lymphocytes, spermatozoa, and platelets) (35) by binding to SV-IV-specific tyrosine kinase-associated plasma membrane receptors (see above). This hypothesis is also consistent with our previously published data that have demonstrated the ability of SV-IV to inhibit in vitro the processes of macrophage antigen presentation and lymphocyte activation following its binding to the plasma membranes of these cells (19, 28).

The cytokine mRNA profiles of lymphocytes and macrophages isolated from the splenocytes populations of mice treated with serovar Typhimurium and SV-IV indicate that SV-IV does not inhibit cytokines (IFN-γ, IL-10, IL-4, IL-5, IL-1, TNF-α, and TGF-β) but, rather, inhibits the serovar Typhimurium-induced rise in cytokine levels. The molecular mechanism underlying the downregulation effect of SV-IV on cytokine gene expression is probably defined by the same mechanism, suggested above, that explains the inhibitory effect of SV-IV on iNOS gene expression.

As we have already mentioned in the introduction, SV-IV is an immunomodulatory protein that activates lymphocytes and macrophages when they are resting, inhibiting them, on the contrary, when they are activated (37). This finding suggests the possibility that SV-IV could play an important role in the maintenance of the homeostasis of the immune system. To play such a role, a molecule is expected to be produced in the right amount only when it is needed, i.e., when the activity of the system being controlled is significantly shifted from the equilibrium point. The production of abnormal levels of this substance for a long time could alter the homeostasis of the system being controlled in such a way as to bring about devastating consequences for the survival of the organism in which this event occurs. In our murine model of experimental serovar Typhimurium infection, an SV-IV-induced inhibitory adjustment of the mouse immune system stimulated by the bacterial invasion is useful if it is appropriate (in terms of time and level) to maintain the balance of the immune response, but if it is not appropriate, it can lead the organism to succumb to the bacterial invasion.

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