involvement of hydroxyl radicals[19] at least, would have been an attractive hypothesis to explain the inhibition of cell-mediated cytotoxicity by ethanol[24], which is an efficient scavenger of hydroxyl radicals. Preliminary experiments done in our laboratory, however, gave no evidence for the production of O_2^-, the main precursor of all oxygen radicals (data not shown).

One of the major mechanisms through which an oxidative stress would lead to cellular damage is the peroxidation of polyunsaturated fatty acids in phospholipids of functional membranes[34]. In fact, the activation of phospholipase A_2 in peripheral blood mononuclear cells[20], and the inhibition of NK activity by inhibition of this enzyme[20,22], together with the findings of Carine and Hudig[23] that arachidonic acid is metabolized via the 5-lipoxygenase pathway in order to function in natural killing may suggest a peroxidative mechanism at least for SCMC. No signs of lipid peroxidation were observed, however, in the course of both SCMC and ADCC in our study. It cannot be fully excluded, however, that oxygen free radicals are produced locally leading to site-specific oxidative damage, including phospholipids, which is not measurable as an overall increase in lipid peroxidation. The fact that glutathione depletion had no effect on either cytotoxicity or lipid peroxidation, however, argues against this hypothesis. Thus, it seems that peroxidative damage is not a mechanism through which lymphocytes mediate their cytotoxicity.

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Enhancement of peritoneal macrophage activity by bovine gamma globulin in mice

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Summary. Mice treated with bovine gamma globulins showed an increased resistance to *Salmonella typhimurium* infection. This phenomenon seems to be bound to an increase of peritoneal macrophage phagocytic activity, as shown by the method of chemiluminescence, in experiements performed on peritoneal macrophages from mice treated with bovine gamma globulin.
Key words. Bovine gamma globulins; macrophages; chemiluminescence; phagocytic activity; *Salmonella typhimurium.*

Previous studies on macrophages have demonstrated that they can be activated by different agents: microorganisms, endotoxins, latex, culture-broth, peptones, plasmatic factors, etc. As indexes of this activation, microbicidal[1] and cytostatic[2] activity, induction of neutral proteinases[3], enhancement of spreading, phagocytosis and pinocytosis[4] and decrease of ectoenzymes[5] were studied. Moreover, secretion of enzymes, proteins, low molecular weight substances and specific factors were also demonstrated[6]. Furthermore, antigens seemed to be more immunogenic when challenged with stimulated rather than unstimulated macrophages. On the other hand, studies on peritoneal macrophages confirmed a different stimulation pattern depending on the various agents[7].

Bovine gamma globulins (BGG) are known as tolerance-inducing agents in several strains of mice; they influence both humoral and cellular immunological factors [8-11]. Amongst others, Lukic[9] clearly showed that differences in susceptibility to induction of tolerance were related to macrophage function in the induction phase of immunity.

Our previous finding that mice pretreated with bovine gamma

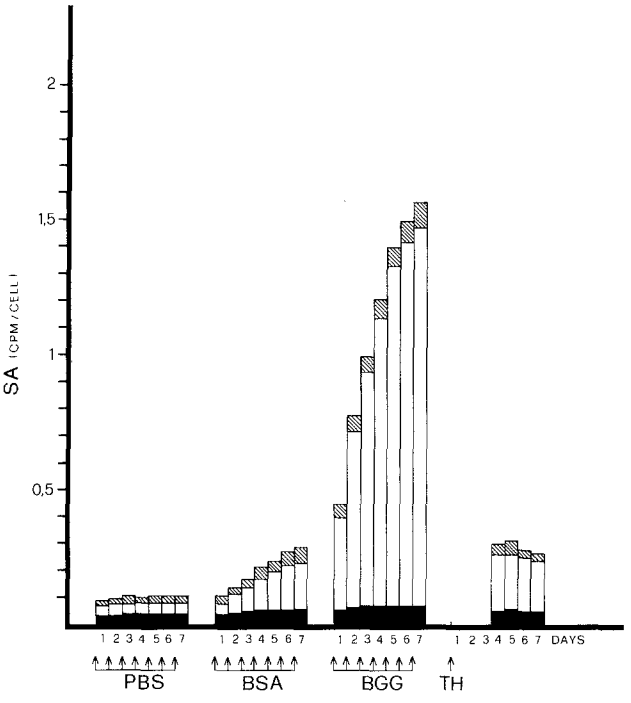

Figure 1. Specific activity (SA) of peritoneal macrophages harvested at indicated times following various treatment schedules and determined with (empty bars) and without (black bars) addition of zymosan. Shadowed part of bars = +SD; arrows = time of each single injection of the respective compound.

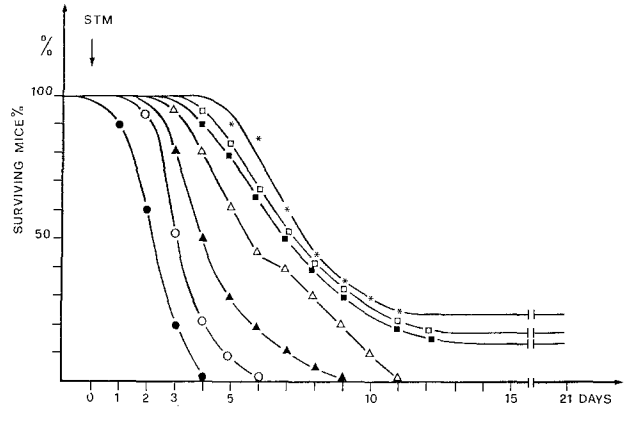

Figure 2. Survival of mice after a single i.p. administration of various doses of *Salmonella typhimurium* (STM). Counts of injected bacteria: (●) 2×10^7, (○) 2×10^6, (▲) 2×10^5, (△) 2×10^4, (■) 2×10^3, (□) 200. (*) 20 bacteria/mouse. Each group consisted of 20 animals.

globulins were more resistant to bacterial infections, together with the fact that bovine gamma globulins did not present any direct antibody-linked specificity against these bacteria[12], let us consider the hypothesis of an aspecific action probably mediated by peritoneal macrophages. To demonstrate this hypothesis, the effect of bovine gamma globulin treatment was studied in two experimental models: a) in vivo in the mice infected with *Salmonella typhimurium;* and b) in vitro by studying mouse peritoneal macrophage functions by means of chemiluminescence (CL) after bovine gamma globulin administration. In fact, chemiluminescence measures macrophage oxidative metabolism directly triggered off by phagocytic stimuli[13].

Materials and methods. Lyophilized bovine gamma globulins (BGG) (Fidia Pharmaceuticals, Padua, Italy) dissolved in phosphate buffered saline (PBS) pH 7.4 were centrifuged at 105,000 × g at 15 °C for 60 min to eliminate any aggregates formed. Bovine serum albumin (BSA) (Eurobio, France) and BGG solutions in PBS were sterilized by means of Millipore Millex sterile filters 0.22 μm and adjusted to a final concentration of 0.5 mg/ml. Modified Krebs-Ringer phosphate medium (KRP) was prepared according to De Sole[14]. Luminol (Sigma) was prepared 1 mM in dimethylsulfoxide. Zymosan (500 mg/ml) from baker's yeast according to Hadding[15], was opsonized by incubation of 0.05 ml of the mother solution for 45 min at 37 °C with 1 ml of heparinized human plasma from healthy donors. Thioglycollate medium (TH) (Difco) was dissolved in H_2O (30 g/l)[16] and sterilized for 15 min at 120 °C.

Swiss mice (weighing 25 ± 3 g) were divided into five groups and kept in the same room with a continuously controlled environment, water and food available ad libitum.

In in vitro experiments five groups, each of 70 mice, were treated i.p. as described in table 1. Ten mice from groups 1, 2, 3 and 4 were killed by decapitation every day, from day 1 to day 7 of treatment. Ten mice from group 5 were killed every day from the fourth to the seventh day after thioglycollate injection. After decapitation, all the blood was allowed to drain out. Recovered suspensions of peritoneal cells, obtained by washing the peritoneum with 5 ml of PBS, were counted by a Coulter Counter.

Macrophage activity was evaluated by a modified method of De Sole et al.[14]. Each scintillation vial contained 0.5×10^6 cells, Luminol 10^{-5} M and KRP medium to a final volume of 1 ml. Chemiluminescence was evaluated at the plateaus both before (activity of resting cells) and after the addition of 0.02 ml of opsonized zymosan (activity of stimulated cells), by a Beta-Counter Packard Tricarb 3385. Specific activity (cpm/cell) was calculated as the ratio of the values obtained at plateaus and the number of cells in each vial. Blanks were obtained in the same way by omitting the addition of cells. Statistical analysis was performed by Student's t-test.

Salmonella typhimurium (STM) group B was used as an antigen for in vivo studies. Cell cultures were allowed to grow on Nutrient Agar (Difco) for 48 h at 37 °C. Agar tubes were rinsed with PBS and recovered suspensions were diluted to obtain 2×10^8 bacteria/ml.

To establish the bacterial dose to be used in further experiments seven groups of animals (each of 20 mice) were infected i.p. with 0.1 ml of STM suspension containing: 2×10^7, 2×10^6, 2×10^5, 2×10^4, 2×10^3, 200, 20, bacteria respectively. The survival of

Table 1. Treatment schedule for in vitro procedure

Group	Treatment	Dose	Injected vol.(ml)
1	None		
2	PBS		0.1
3	BSA	50 μg/day	0.1
4	BGG	50 μg/day	0.1
5	TH	30 mg (*)	1.0

(*) Single administration.

Table 2. Mice peritoneal cell recovery. The table reports the number of cells/ml recovered from mouse peritoneum and differential cell count (mean ± SEM) on the seventh day of treatment (four days after the challenge for TH group)

Group	Treatment	Peritoneal cells cells/ml (mean ± SEM) (10^6)	Monocytes + macrophages (%)
1	None	2.1 ± 23%	21 ± 1
2	PBS	2.3 ± 27%	22 ± 1
3	BSA	2.5 ± 22%	19 ± 1
4	BGG	2.3 ± 21%	21 ± 1
5	TH	7.5 ± 19%	63 ± 3

Statistical analysis vs the control group, carried out by Student's t-test, showed no significant differences between the groups except in the case of the TH group ($p < 0.05$).

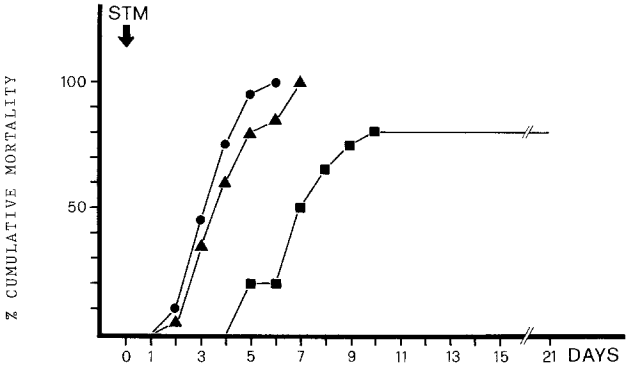

Figure 3. Comparison of mortality rates in PBS (●), BSA (▲) or BGG (■) pretreated mice after i.p. administration of *Salmonella typhimurium* (2×10^6 cells/mouse).

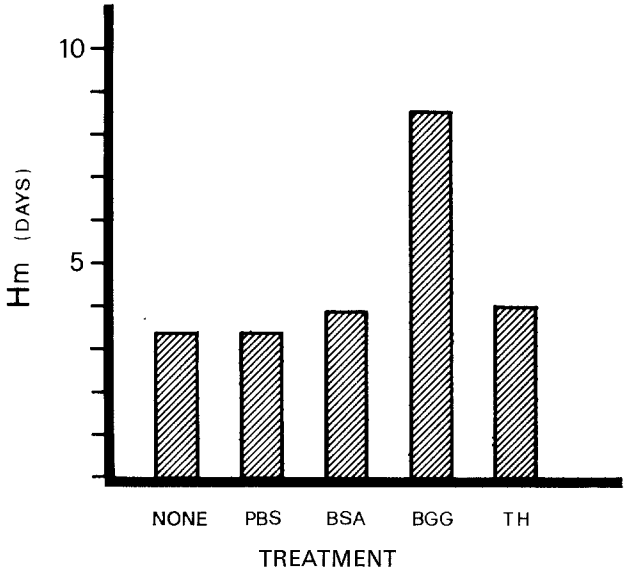

Figure 4. Average survival time expressed by harmonic mean (Hm) of differently treated mice after infection with *Salmonella typhimurium* (20 animals each group).

the animals was controlled every day for 15 days after infection. To evaluate the effect of BGG treatment, five groups of 20 animals each were treated i.p. as reported in table 1. After seven days of treatment groups 1–4 were infected by a single i.p. injection of 0.1 ml containing 2×10^6 bacteria. The daily treatments were carried out after STM injection until the death of the animals or the end of the experiment. Group 5 had an STM injection (2×10^6 bacteria/mouse) four days after thioglycollate treatment. The survival of the animals was checked every day for 15 days after infection. Average survival time in days was expressed in terms of the harmonic mean (Hm) according to Kierszenbaum[17].

Results. In vitro. No significant differences were observed either in cell number for each group of mice considered, or in differential cell counts carried out by the non-specific esterase staining method[18], with the exception of the TH group, which showed an increased total number of peritoneal cells and macrophage fraction (table 2). The neutrophil fraction was found to be lower than 2% of the total number of cells, in all groups studied.

Figure 1 shows time courses of CL specific activity of both resting and stimulated cells, after PBS, BSA, BGG, and TH treatments. The results for the untreated group were always similar to those obtained for the PBS treatment group. No significant difference in resting cell activities was found between any of the groups at any time. After stimulation with zymosan, macrophages from BGG treated mice showed a very sharp time-dependent increase in their specific activities, with a maximum on the seventh day of treatment. Stimulated cell activities in PBS, BSA, TH groups were found to be higher than resting cell activities but much lower when compared to the BGG group.

In vivo. In figure 2 the dose-effect curves for STM infection are reported. Up to 2×10^6 bacteria/mouse, 100% of mice died of sepsis within 4–6 days after infection. With doses of 2×10^4 down to 20 bacteria/mouse, death by sepsis was determined in 20–30% of the animals only, while most of the mice died of mouse typhoid disease. The dose of 2×10^5 bacteria/mouse could be considered as a boundary dose between sepsis and typhoid disease[19]. On this basis the dose of 2×10^6 bacteria/mouse was chosen to perform further experiments. In figure 3 the effect of the different treatments in mice infected with a dose of 2×10^6 bacteria/mouse is reported. Mouse survival after BGG treatment appears to be higher with respect to BSA, PBS and TH treatments. A comparison of the data reported in figures 2 and 3 shows that the mice treated with BGG died mostly of typhoid disease as did untreated mice infected with a dose ten thousand times lower. Figure 4 reports the average survival time, expressed by the harmonic mean (Hm), of the differently treated groups of mice. BGG treatment gave a Hm value twice as high as those obtained in the control groups.

Our study was undertaken to focus on the relationship between bovine gamma globulin administration and phagocytic activity of peritoneal mouse macrophages.

Results obtained in vitro by chemiluminescence evaluation showed an enhanced macrophage activity in BGG treated mice, as compared to control or differently treated groups (BSA, TH). However, different treatments are all unable to induce macrophage stimulation per se in respect to the untreated mice: in fact their resting cell activities are not significantly different. After the addition of zymosan, macrophages from BGG treated mice showed a 20-fold enhancement of their phagocytic capability measured by CL response with respect to control groups. Thus BGG can not be considered as true 'stimulators' but as activators of the intrinsic phagocytic capabilities of macrophages, which become more aggressive against external agents.

The other model we chose was a bacterial (STM) infection in vivo. Mouse response to STM challenge is dose-dependent. If the number of bacterial cells injected is high, the animals usually die within four days of sepsis, while if a low dose is used, death from typhoid disease occurs after about a week. The number of bacterial cells injected in our experimental model was a sepsis-inducing dose. The in vivo results showed a highly significant longer survival time in BGG treated mice; thus BGG treatment seems to be very effective in the first phase of infection, protecting mice against sepsis.

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Na fluxes in human mononuclear leucocytes

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Summary. Unidirectional ^{22}Na fluxes were investigated in human peripheral mononuclear leucocytes. Ouabain inhibited about 60% of Na efflux and the addition of bumetanide further reduced Na efflux rate by about 45%, suggesting the presence of a transport pathway capable of extruding Na against its gradient. Prostaglandins E_1 and E_2 and exogenous cAMP were found to be potent inhibitors of the bumetanide-sensitive Na efflux without affecting the ouabain-sensitive or the ouabain and bumetanide resistant Na effluxes.
Key words. Na transport; lymphocytes; PGE.

The maintenance of cell Na and K levels is thought to be dependent on a balance between an active transport system that creates and maintains gradients and passive fluxes that dissipate these gradients. In most cells, the ouabain-sensitive Na, K pump is a major determinant of Na and K gradients across cell membranes. Recently, systematic study of ouabain-insensitive fluxes revealed the presence of a transport pathway sensitive to inhibition by derivatives of 2 sulfamoylbenzoic acid, such as furosemide, bumetanide, piretanide[1,2]. While various cell types exhibit furosemide-sensitive Na and K fluxes, human and avian erythrocytes have proved to be especially suitable for their study and various investigators showed it to be mediated by the Na, K, Cl cotransport system[3] and to be independent of other transport systems such as the Na, K-pump, Na-Na exchange or Na, Cl pathway[2,4,5]. Furthermore the Na, K, Cl cotransport has been shown to be regulated by various factors. This system appears to be sensitive to changes in metabolic status[6], as well as shifts in cAMP levels[7,8] or hypertonicity.

The erythrocyte has proven to be a very useful cell for studying the kinetics of the furosemide-sensitive transport system or its involvement in the regulation of cell volume. However, questions concerning the involvement of a cellular cation in cell growth, cell cycle or immunological response cannot be investigated using this cell. To date, analysis of cation transport in the human peripheral lymphocyte is far from complete. A Na, K pump has been identified in human lymphocytes[9,10], likewise a Na, H exchange and a K channel have been described[11]. These cation transport systems are less well characterized than their counterparts in the erythrocyte mainly because work with such cells is hampered by complications owing to a limited number of cells being available, and the need for delicate cell handling.

In the present study we have investigated Na transport in human peripheral blood mononuclear cells (PMC). We have described the presence of a ouabain-sensitive Na pump and a bumetanide-sensitive Na transport system. Furthermore, it has been shown that prostaglandins E_1 and E_2 and db cAMP regulate this latter system.

Materials and methods. Human peripheral blood mononuclear cells (PMC) were isolated by a Percoll gradient centrifugation technique[12]. PMC were obtained from plateletphoresis-residues following a method described by Segel et al.[13]. Cells were suspended at a cell concentration of 10^7 cells/ml in a PBS medium containing (mM): 137 NaCl, 3 KCl, 8.1 Na_2HPO_4, 1.2 KH_2PO_4, 10 glucose, 0.7 $CaCl_2$, 0.5 $MgCl_2$, 0.4 $MgSO_4$, pH 7.4 at 37°C. Pyruvate 1 mM and albumin 1 mg/ml were added to the media and kept at 37°C for 60 min prior to assay. Addition of Trypan blue to the medium revealed that 95% of cells excluded the dye. Cell viability markedly decreased when suspensions with a cell number $> 3 \times 10^7$ cells/ml were kept for 12 h at 37°C in PBS medium. Cell counting was performed using a Malassez type

Table 1. The effect of ouabain and bumetanide on ^{22}Na efflux rate from peripheral mononuclear cells

Experiment No.	^{22}Na efflux rate (min^{-1}) PBS medium	PBS + ouabain (10^{-3}M)	PBS + ouabain (10^{-3} M) + bumetanide (10^{-4} M)
1	0.072	0.015	0.008
2	0.040	0.019	0.012
3	0.055	0.027	0.014
4	0.051	0.025	0.013
5	0.037	0.019	0.010
6	0.066	0.029	0.014
Mean ± SD	0.054 ± 0.014	0.022 ± 0.005*	0.012 ± 0.002**

^{22}Na loaded human peripheral mononuclear cells were incubated in phosphate buffered medium (PBS). Triplicate samples were taken at different time intervals and the rate constant k was calculated by linear regression analysis of values obtained using the equation $A_t = A_0 e^{-kt}$, where A_t: activity at time t, A_0: activity at time zero. Statistical significance was assessed using Student's t-test. *$p < 0.01$ when compared to values obtained in PBS medium; **$p < 0.001$ when compared to PBS+ouabain medium.