C5a receptor and thymocyte apoptosis in sepsis

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ABSTRACT

In sepsis, apoptosis occurs in many different organs. The mediators responsible for induction of apoptosis are not clearly known, although there are some suggestions that C5a and the C5a receptor (C5aR) might be directly linked to apoptosis. In the cecal ligation/puncture (CLP) model of sepsis in rats, apoptosis occurs early in a variety of organs, especially in the thymus. We demonstrate that thymocytes from normal rats show specific, saturable, and high affinity binding of ^{125}I-labeled recombinant rat C5a. C5a binding to thymocytes was significantly increased 3 h after CLP and also when thymocytes from normal rats were first incubated *in vitro* with lipopolysaccharide (LPS) or IL-6. The expression of C5aR mRNA in thymocytes was markedly increased 3, 6, and 12 h after CLP and increased similarly when normal thymocytes were first exposed to LPS or IL-6 *in vitro*. Thymocytes obtained 2 or 3 h after CLP and exposed *in vitro* to C5a, but not normal thymocytes, underwent increased apoptosis, as demonstrated by annexin-V binding, coinciding with increased activation of caspases 3, 6, and 8. These data provide the first direct evidence that in the early onset of sepsis, increased expression of C5aR occurs in thymocytes, which increases their susceptibility to C5a-induced apoptosis.

Key words: cecal ligation/puncture • caspases • thymus • complement

The complement activation product, C5a, has been shown to play an important inflammatory role in rodents in the early phases of sepsis following cecal ligation/puncture (CLP) or after infusion of lipopolysaccharide (LPS) (1, 2). Besides its strong chemotactic activity, other effects are known, such as release from phagocytic cells of granular enzymes, production of superoxide anion, histamine release from mast cells, vasodilatation, increased vascular permeability, and smooth muscle contraction (3–7). The responses to C5a are mediated by a pertussis toxin-sensitive G-protein-linked seven-transmembrane C5a receptor (C5aR), which belongs to the superfamily of rhodopsin-type receptors (8, 9).

In recent years, the receptor for C5a has been shown to be present in many different organs (e.g., liver, kidney, lungs, brain) (10–14), although C5aR was originally considered to be confined to myeloid cells (15). Little is known about the functional importance of C5aR on nonmyeloid cells. During the development of sepsis, most organs undergo apoptosis, the thymus being especially sensitive to this process during sepsis (16, 17). We have recently reported that treatment of CLP rats with anti-C5a

protects thymocytes from apoptosis, suggesting a direct or indirect role for C5a in thymocyte apoptosis (18). A C5a or C5aR fragment with agonistic activity induces rapid elevation of nuclear c-fos in human neuroblastoma cells, resulting in DNA fragmentation (19, 20). Aside from these findings, there has been no other direct evidence for the ability of C5a to induce apoptosis. Therefore, we investigated changes in C5aR mRNA expression and ^{125}I-C5a binding in rat thymocytes before and during sepsis, and the ability of exogenous C5a to induce thymocyte apoptosis *in vitro*, after the induction of C5aR in the onset of sepsis. Our findings provide direct evidence for the ability of C5a to induce apoptosis in thymocytes, provided C5aR has been up-regulated.

MATERIALS AND METHODS

Recombinant rat C5a and human C5a

Recombinant human C5a (hu C5a) was obtained from Sigma (St. Louis, MO). Recombinant rat C5a (rrC5a) was produced in our laboratory, using the pGEX expression vector for GST gene fusion (Amersham Pharmacia Biotech, Piscataway, NJ) (18).

Experimental sepsis induced by CLP

Five- to eight-week-old specific pathogen-free male Long-Evans rats (Harlan, Indianapolis, IN) were used for all studies. Anesthesia was achieved by i.p. injection of Ketamine (20 mg/100 mg body weight). In the CLP model, approximately one-third of the cecum was ligated through a 2-cm abdominal midline incision. The ligated part of the cecum was punctured through with a 21-gauge needle. After repositioning of the bowel, the abdomen was closed in layers, using a 4.0 surgical suture (Ethicon, Somerville, NJ) and metallic clips. Sham animals underwent the same procedure without ligation or puncture of the cecum.

Thymocyte isolation and in vitro stimulation

At different time points (0, 3, 6, and 12 h) following induction of CLP, animals were killed and the thymus was removed surgically immediately thereafter and placed on ice. Thymocytes were isolated according to the method of Morris et al. (21). After cell counting, the thymocyte suspension was diluted to 4–5 million cells/ml using Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker, Walkersville, MD) containing 0.1 % bovine serum albumin (BSA). Thymocytes were then stimulated with different concentrations of rat or human C5a, LPS, or IL-6 at 37°C.

Binding studies

rrC5a was labeled with ^{125}I, using the chloramine method, as described previously (22). This protocol allowed gentle oxidation and resulted in intact chemotactic activity for the C5a preparation (23). Rat thymocytes were isolated (as previously described) and suspended in microcentrifuge tubes (1.5 ml) in the binding buffer Hanks' balanced salt solution (HBSS) and 0.1% BSA at a final concentration of 4×10^7 cells/ml. The cell suspensions (200 µl) were then incubated at 4°C for 20 min in the presence of ^{125}I-rrC5a (specific activity of 30–40 µCi/µg). The cell suspension was then layered over 20% sucrose and sedimented by centrifugation (11,000g) in a swinging bucket rotor for 2 min. After centrifugation, the tubes were frozen at –80°C, and the tips (containing the cell pellets) were cut off to determine the cell-

bound ^{125}I-rrC5a, using a gamma counter (1261 Multiγ, Wallace, Gaithersburg, MD). Results were then expressed as counts per minute, as a function of the concentration of ^{125}I-rrC5a, in order to establish the saturation curve.

RNA isolation and detection of C5aR mRNA by reverse transcriptase-polymer chain reaction (RT-PCR)

Total RNA was isolated with the Trizol method (Life Technologies, Rockville, MD) according to the manufacturer's directions. Freshly isolated thymocytes were stimulated with LPS and IL-6 at various concentrations for 2 h at 37°C before total RNA was isolated. RNA was also isolated from thymocytes from rats 3, 6, and 12 h after induction of CLP. Digestion of any contaminating DNA was achieved by treatment with RQ1 Rnase-free DNase (Promega, Madison, WI).

Reverse transcription was performed with 1 µg RNA, using the Superscript II RNase H⁻ Reverse Transcriptase (Gibco BRL, Grand Island, NY) according to the manufacturer's protocol. PCR was then performed with primers for C5aR: 5' primer 5'-TAT AGT CCT GCC CTC GCT CAT-3' and 3' primer 5'-TCA CCA CTT TGA GCG TCT TGG-3'. The primers were designed for a 409-bp cDNA amplification in the middle region of the rat C5a receptor cDNA (position 373 to 781). The primers for the "housekeeping" gene GAPDH were 5' primer 5'-GCC TCG TCT CAT AGA CAA GAT G-3' and 3' primer 5'-CAG TAG ACT CCA CGA CAT AC-3'. After a "hot start" for 5 min at 94°C, 35 cycles were used for amplification with a melting temperature of 94°C, an annealing temperature of 60°C, and an extending temperature of 72°C, each for 1 min, followed by a final extension at 72°C for 8 min. The RT-PCR product was confirmed by electrophoresis of samples in 1.2% agarose gel. Control experiments were performed with the samples in which RT was not added to rule out contaminating DNA being responsible for results. PCR was performed from rat thymocyte mRNA, using different cycle numbers for C5aR and GAPDH, to ensure that DNA was detected within the linear part of the amplifying curves for both primers.

Apoptosis assay and flow cytometry

Thymocyte apoptosis was detected using the Annexin V Fluos staining kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. In this assay, Annexin V (Ax) is conjugated to FITC and propidium iodide (PI). Approximately 4×10^6 cells/ml were used for each assay. After they were stained for 12 min, the cells were immediately placed on ice and analyzed with a flow cytometer (Coulter, Miami, FL), using 488-nm excitation and a 525-nm bandpass filter for FITC and a 620 nm filter for PI detection. Data were then analyzed with WinList computer software (Verity, Topsham, ME). Electronic compensation of the instrument was carried out to exclude overlapping areas of the two emission spectra. Cell debris was eliminated by gating according to side-scatter and forward-scatter detection. The regions for Ax-positive only, PI-positive only, double-positive, and double-negative cells were created by using single-parameter analysis with Ax-FITC or PI.

Caspase activity assay

Caspase 3, 6, and 8 activities were measured by a fluorometric assay (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer's instructions. In brief, thymocytes were isolated 3 h after CLP and then stimulated with nothing, with huC5a, or with rrC5a at 37°C for 3 h. Cell lysates from 8×10^6

thymocytes were incubated with the following caspase substrates (50 μM final concentration): Ac-DEVD-AFC (caspase 3), Ac-VEID-AFC (caspase 6), and Ac-IETD-AFC (caspase 8). After the cell lysates were incubated at 37°C, free AFC (7-amino-4-trifluoromethyl coumarin) was detected on a Cytofluor II plate reader (Millipor, Bedford, MA) with a 400-nm excitation and a 505-nm emission filter. Caspase activation was expressed as the relative increase compared with the control values from nonstimulated thymocytes.

RESULTS

Binding of ^{125}I-rrC5a on thymocytes

To detect C5aR in rat thymocytes from normal rats, we used binding experiments with ^{125}I-rrC5a as described previously. Figure 1A shows the saturation curve, using 1–1000 pM ^{125}I-rrC5a. Nonspecific binding was assessed in the presence of 50-fold excess of nonlabeled rrC5a for the different concentrations of ^{125}I-rrC5a. Saturation was reached at ~500 pM. With increasing amounts of nonlabeled rrC5a, binding of ^{125}I-rrC5a to thymocytes could be competed against, as demonstrated in Figure 1B. Between 10^{-14} and 10^{-11} M nonlabeled rrC5a, the binding signal achieved with 200 pmol ^{125}I-rrC5a could be reduced by ~50%.

Increased ^{125}I-rrC5a binding to LPS- and IL-6-treated thymocytes and to thymocytes from CLP rats

To determine whether enhanced binding of C5a occurred in rat thymocytes during the onset of sepsis, thymocytes were isolated 3 h after CLP and compared with normal thymocytes. Figure 2A demonstrates significantly increased binding of ^{125}I-rrC5a to thymocytes obtained from rats 3 h after CLP. To further investigate mediators possibly responsible for these results, we isolated thymocytes from normal rats and stimulated them *in vitro* with LPS and IL-6 at various concentrations for 3 h at 37°C, because these agonists are known to cause increased expression of C5aR in rat hepatocytes (10). Figures 2B and 2C demonstrate that each mediator, in a dose-dependent manner, was capable of significantly increasing ^{125}I-rrC5a binding to thymocytes. At concentrations of 100 ng/ml IL-6 and 500 ng/ml LPS, binding appeared to be maximally increased.

Detection of mRNA for C5aR with RT-PCR

To assess changes in mRNA for C5aR in rat thymocytes and assess conditions that might cause its increase, we performed RT-PCR experiments for C5aR mRNA as described previously. mRNA for C5aR in thymocytes obtained from normal rats could be faintly detected in the absence of any other manipulation (Fig. 3A, lane 1, ctrl). Thirty-five cycles of amplification were required to detect a faint band, suggesting a fairly low copy number of C5aR mRNA under these conditions. To investigate whether C5aR could be induced in thymocytes after exposure to bacterial endotoxin (LPS) or to IL-6, we incubated thymocytes from normal rats, with LPS (1–100 ng/ml) from *Escherichia coli* or with recombinant rat IL-6 (1–100 ng/ml) for 2 h at 37°C. Figure 3A shows the dose-dependent increases in mRNA for C5aR. Compared with nonstimulated (ctrl) thymocytes, LPS at 1 ng/ml and IL-6 at 100 ng/ml clearly increased expression of C5aR mRNA within 2 h of stimulation. Above a concentration of 1 ng LPS/ml, there was a progressive reduction in mRNA for C5aR, whereas with IL-6, there was a progressive increase in expression from 1 to 100 ng IL-6/ml.

To address the question of whether mRNA of C5aR is induced in thymocytes during sepsis, we isolated thymocyte RNA at 3, 6, and 12 h after CLP. The copy number for C5aR mRNA was clearly increased as early as 3 h after CLP (Fig. 3, lower frame, lane 2), suggesting rapid up-regulation of C5aR in the early period of sepsis. After 6 h, the increase in C5aR mRNA was similar to the findings at the 3-h time point. However, 12 h after induction of CLP, the increase of mRNA for C5aR was even more intense. This is a time when thymocyte apoptosis is well advanced (18, 25). These data indicate that mRNA for C5aR is increased in the early period of CLP-induced sepsis and that C5aR protein is correspondingly increased, as indicated by increased ^{125}I-C5a binding.

Induction by C5a of thymocyte apoptosis

Because we were recently able to show that anti-C5a treatment could successfully reduce thymocyte apoptosis during sepsis (18), we investigated whether thymocyte apoptosis could be induced by direct exposure to C5a under conditions in which C5aR expression has been increased. Rats were sacrificed, and thymocytes were obtained at various time points (0, 2, 3, 6, 12, and 24 h) after CLP. They were then incubated with either human C5a or rrC5a (at 37ºC for 0.5, 1.0, or 2.0 h). Apoptosis was measured by binding of annexin-V to thymocytes, as determined by flow cytometry. Preliminary experiments showed significant apoptosis in thymocytes obtained 2 and 3 h after CLP, after incubation with huC5a or rrC5a for 30 min at 37ºC (data not shown). For time points beyond 3 h following CLP, the extent of apoptosis was already too advanced to detect any effect of added C5a (data not shown). Using thymocytes 2 or 3 h after CLP, we conducted experiments, stimulating thymocytes with different concentrations of huC5a and rrC5a (between 1 and 200 nM). The strongest apoptotic outcome resulted from 100 nM huC5a and 30 nM rrC5a. Figure 4A summarizes the increase in apoptosis and necrosis of thymocytes after the addition of 30 nM rrC5a or 100 nM huC5a relative to no addition of C5a ("none"), using thymocytes 2 or 3 h after the onset of CLP. Figure 4B shows the results of a typical flow cytometry experiment. Exposure to either huC5a or rrC5a induced apoptosis of thymocytes from CLP rats, but not from normal rats (data not shown).

C5a-induced activation of caspases in thymocytes

To extend the findings described previously, we investigated the ability of C5a to induce caspase activity in thymocytes. Thymocytes were isolated from rats 3 h after induction of CLP and then incubated at 37°C with 100 nM huC5a or 30 nM rrC5a or in the absence of C5a for 30 minutes. Afterwards, cells were lysed, and lysates were incubated with the fluorescently tagged substrates. Enzymatic activity was detected by increases in cleavage of caspase substrates, as determined by fluorometry. Results were expressed as relative increases compared to the non-stimulated control group. Figure 5 shows the results of a representative experiment in which each sample was assayed in triplicate. In terms of increasing caspases 3, 6, and 8 activity, 30 nM rrC5a was more effective than 100 nM huC5a. In the case of caspases 3 and 6, the increase was 40% and 37%, respectively, following the addition of rrC5a stimulation. Caspase 8 activity was very low in general, but thymocyte contact with rrC5a caused it to increase by 27%. In thymocytes from normal rats, incubation with rrC5a (30 nM) or huC5a (100 nM) did not result in caspase activation (data not shown).

DISCUSSION

There is increasing evidence that C5a may play an important role in events linked to the early stages of experimental sepsis, as shown by protective effects of anti-C5a (2, 24–26). In these studies, anti-C5a treatment was instituted in the early period (1–6 hours) after CLP to achieve protective effects. These studies suggested that many of the pathophysiological changes in experimental sepsis could be ascribed to early production of C5a. As mentioned previously, C5a is responsible for many pathophysiological changes (3–7). These effects are achieved through the interaction of C5a with a seven-transmembrane G-protein-linked C5aR, which was originally detected in myeloid cells (15) and has now been detected in several different organs (10–14). On the basis of recent studies in rodents, C5a is considered to play an important role in the early phases of CLP-induced sepsis (27). We recently demonstrated a dramatically protective effect of anti-C5a treatment on thymocyte apoptosis during CLP-induced sepsis (18). In these studies, we were not successful in inducing apoptosis *in vitro* by incubation of normal thymocytes (rat) with rrC5a. We therefore measured in thymocytes the presence of mRNA for C5aR and binding of ^{125}I-labeled C5a, because currently no available antibody will detect rat C5aR protein. C5aR protein has been described in human T cells (28).

Binding studies have shown specific, saturable, and high affinity binding of C5a to rat thymocytes (Fig. 1), implying the presence of C5aR, even though its density on thymocytes is probably low. When thymocytes from normal rats were stimulated *in vitro* with LPS or IL-6, binding of ^{125}I-rrC5a increased significantly. Thymocytes from rats 3 h after CLP also showed significantly increased binding. These data provide evidence that C5aR is present on thymocytes and that it is up-regulated during the development of sepsis, perhaps by mediators that are known to play an important role during the onset of sepsis in the CLP model.

We extended our findings by RT-PCR experiments to evaluate C5aR mRNA. We found in thymocytes from normal rats that the copy number of mRNA for C5aR was very low. With 35 amplification cycles, it was possible to find a weak band, as detected in 3 (out of 10) separate experiments. C5aR mRNA levels in normal thymocytes rose appreciably after 2 h of stimulation with IL-6 or LPS. It is interesting that at higher LPS concentrations, mRNA levels for C5aR fell, perhaps due to unstable mRNA. In contrast, *in vitro* incubation of thymocytes with IL-6 showed a direct dose-response relationship for mRNA for C5aR. Because IL-6 is known to be up-regulated at the onset of CLP-induced sepsis (27), we investigated whether mRNA for C5aR in thymocytes was increased during sepsis. We were able to show that, as early as 3 h following CLP, there was clearly increased mRNA for C5aR on thymocytes (Fig. 3) and that this was associated with increased binding of C5a to thymocytes obtained 3 h after CLP (Fig. 2), strongly implying up-regulation of C5aR during sepsis. The levels of C5aR mRNA were even more intense at 12 h. Binding studies, together with the RT-PCR results, strongly suggest that C5aR is induced in the early onset of sepsis. An antibody to rat C5aR is currently not available, precluding direct measurements of C5aR protein on rat thymocytes.

To address the question of whether C5a can directly induce apoptosis in thymocytes under conditions in which the C5aR is up-regulated, we investigated thymocyte apoptosis and thymocyte mRNA for C5aR in CLP animals at different time points. Previous studies have shown that a C5a or C5aR fragment with agonist activities induces apoptosis in human neuroblastoma cells (19, 20). We were able to show for the first time that C5a can directly induce apoptosis in thymocytes obtained 2 and 3 h after CLP in rats. In sham animals (operated but absent CLP), no such evidence could be found. Corticosteroids are known to

induce apoptosis in thymocytes (29, 30), and Fas-ligand also plays an important role, in contrast to endotoxin and tumor necrosis factor α, which are said to evoke no direct apoptotic response (31–33). The extent to which C5a contributes to apoptosis during CLP requires additional investigation. Our recent studies have shown that treatment of CLP rats with anti-C5a greatly reduces the amount of thymocyte apoptosis (18). The data presented here show that C5a incubated with thymocytes obtained 2 h after CLP induced substantial levels of apoptosis. The extent of *in vitro* induction of apoptosis likely depends on the cell surface content of C5aR that are not occupied by their ligand and internalized. We conducted experiments with a cyclic C5aR antagonist to block C5a-mediated thymocyte apoptosis *in vitro*. Unfortunately, this antagonist showed agonist activity in our experiments and induced apoptosis in CLP thymocytes (data not shown). We can not rule out the possibility that a costimulatory mechanism may be involved in C5a-induced thymocyte apoptosis *in vitro*, possibly related to a "priming" of thymocytes during the onset of CLP-induced sepsis by proinflammatory mediators (e.g., LPS, cytokines).

We were able to demonstrate in CLP thymocytes that C5a induced increases in caspases 3, 6, and 8 enzymatic activities following thymocyte exposure to rrC5a or huC5a. Different caspases are activated in sepsis-induced thymocyte apoptosis (18, 34). A recent study has unexpectedly shown a neuroprotective effect of C5a mediated through caspase-3 inhibition (35). Our findings contrast with those of this study. Thymocyte apoptosis occurring during sepsis is thought to be beneficial in terms of reducing an overwhelming immune response (36), although the loss of lymphoid cells during sepsis is generally considered to lead to potentially threatening immunosuppression.

In this study, we provide evidence for the induction of C5aR in rat thymocytes during sepsis, as demonstrated by C5aR RT-PCR and C5a binding studies. We also demonstrate the ability of IL-6 or LPS to cause similar changes *in vitro* in normal thymocytes. Under conditions in which C5aR is up-regulated, C5a appears able to directly induce apoptosis in thymocytes. To our knowledge, this is the first time in CLP-induced sepsis, that C5aR induction is demonstrated. Under these conditions, C5a can then directly induce apoptosis in thymocytes. This study suggests not only a new role for C5a in sepsis, but also the importance of C5aR induction during sepsis. C5aR may well be a target for drug therapy to control undesirable apoptotic pathophysiological changes in the early stages of sepsis.

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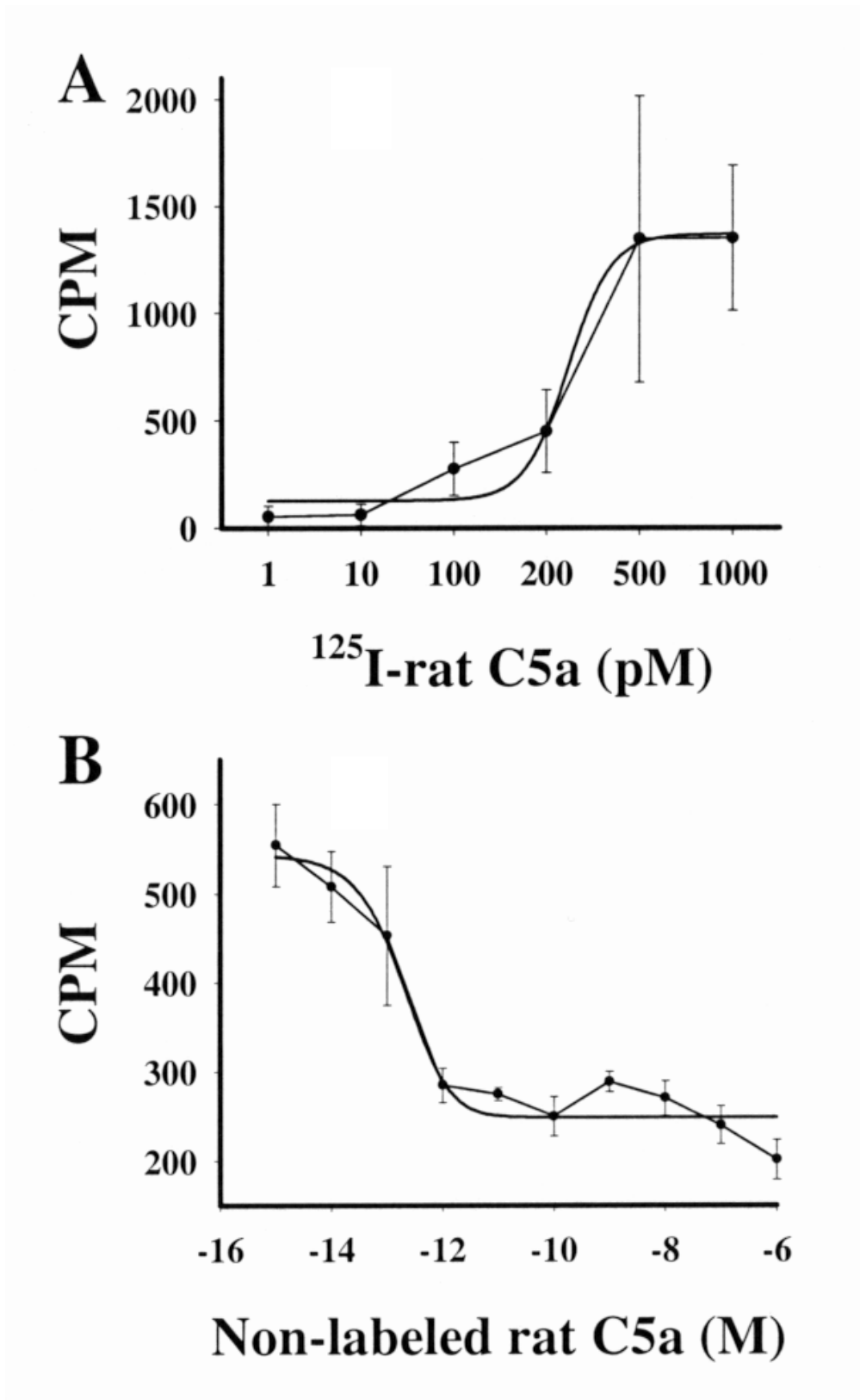
Fig. 1

Figure 1. ^{125}I-rrC5a binding to thymocytes from normal rats, expressed as counts per minute (CPM). A) Binding saturation occurred at ~1 nM. **B)** Competition of binding achieved with 200 pM ^{125}I-rrC5a by increasing amounts of nonlabeled rrC5a. Competition was achieved between 10^{-14} and 10^{-10} M C5a. Results are from three to nine independent experiments carried out for each data point, which represents quadruplicate samples.
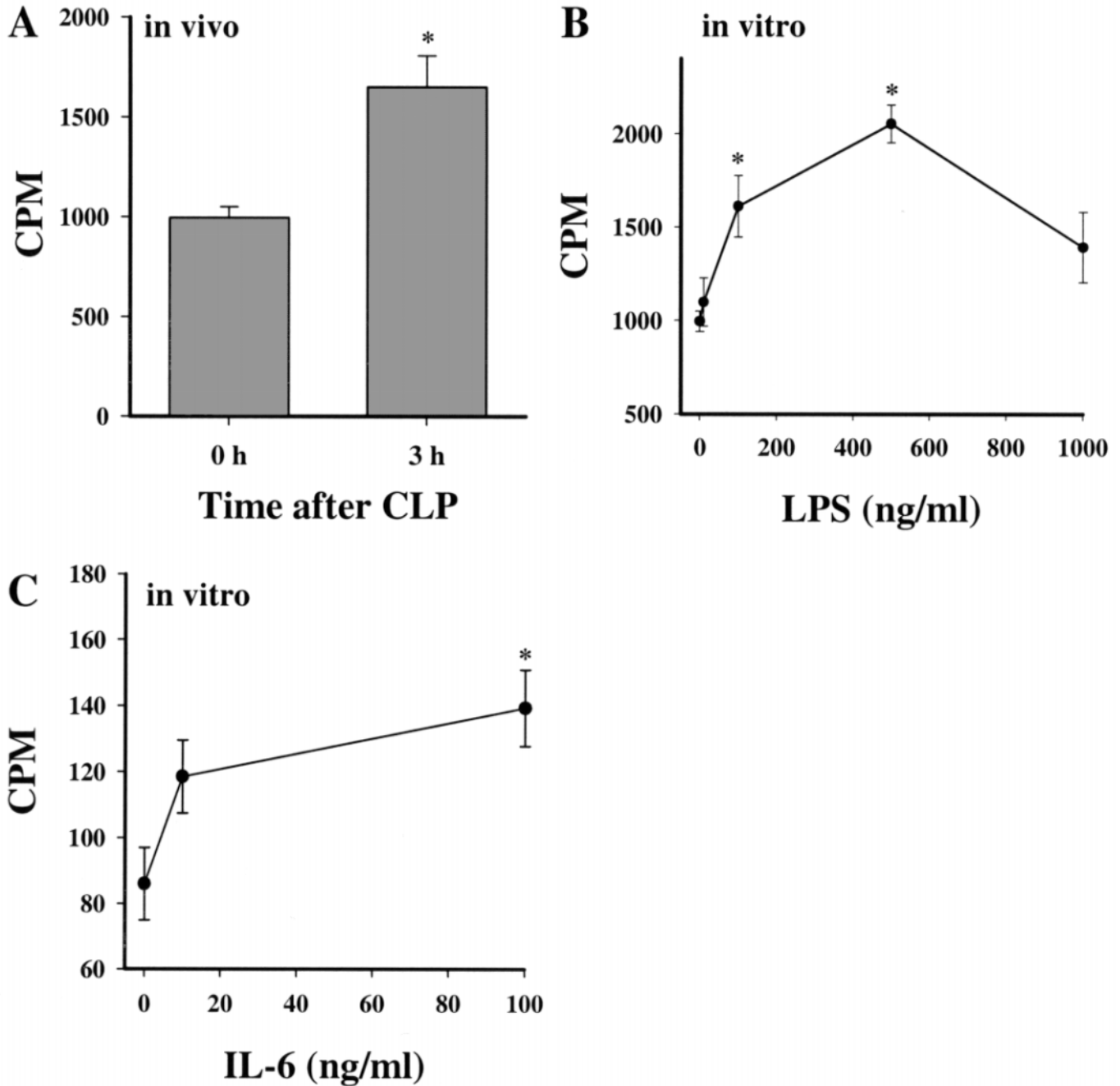
Fig. 2

Figure 2. ^{125}I-rrC5a binding to rat thymocytes obtained 3 h after cecal ligation/puncture (CLP) (A) and after lipopolysaccharide (LPS) or IL-6 stimulation *in vitro* (B, C). *Significantly increased binding compared with the nonstimulated controls. Data are representative of three to six independent experiments, carried out in quadruplicate samples.
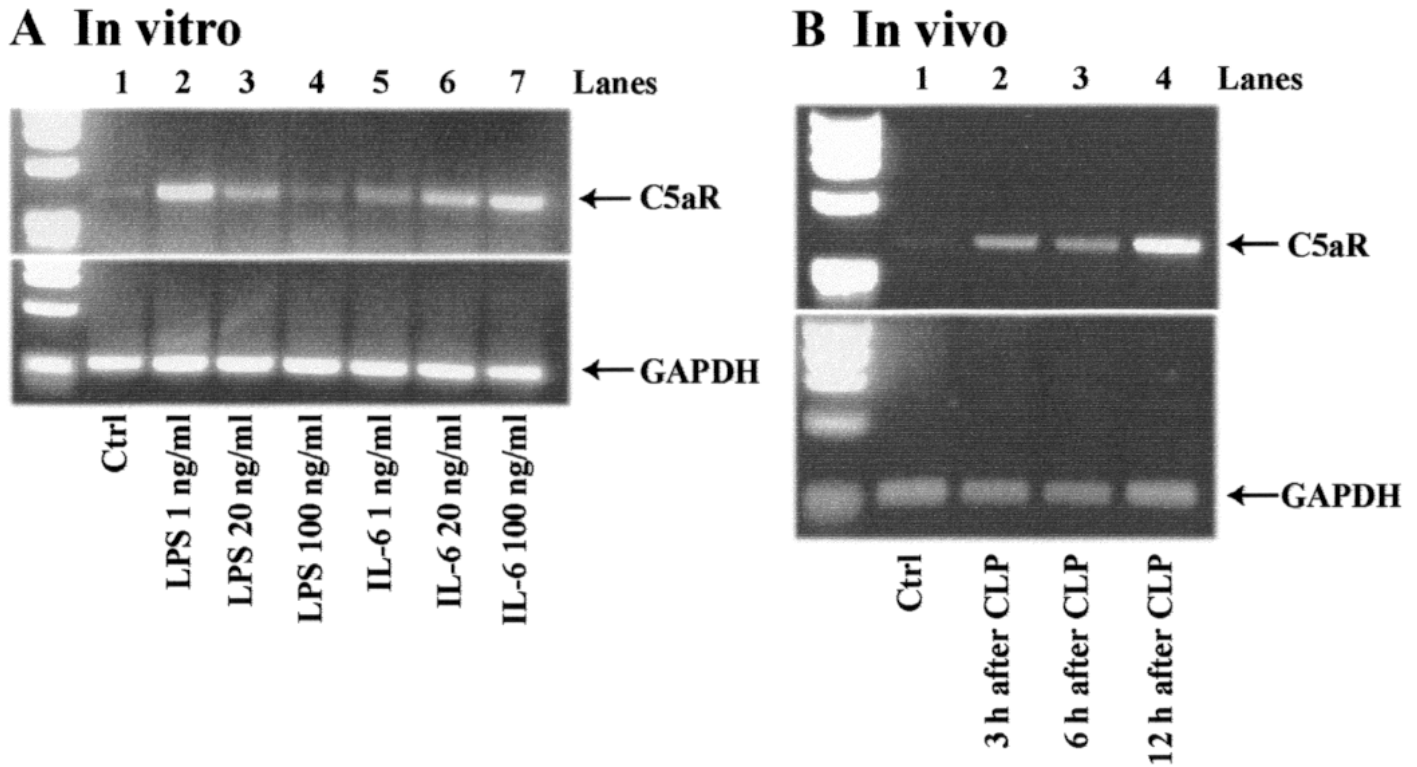
Fig. 3

Figure 3. Semiquantitative reverse transcriptase-polymer chain reaction analysis for C5aR mRNA in rat thymocytes. A) Thymocytes from normal rats were stimulated with lipopolysaccharide (LPS) and IL-6 *in vitro* at different concentrations. **B)** Induction of C5aR mRNA *in vivo* as early as 3 h and as long as 6 and 12 h after cecal ligation/puncture (CLP). Equal loading conditions were demonstrated with bands for GAPDH-mRNA production (lower panels in both frames). Data are representative of results from two independent experiments.
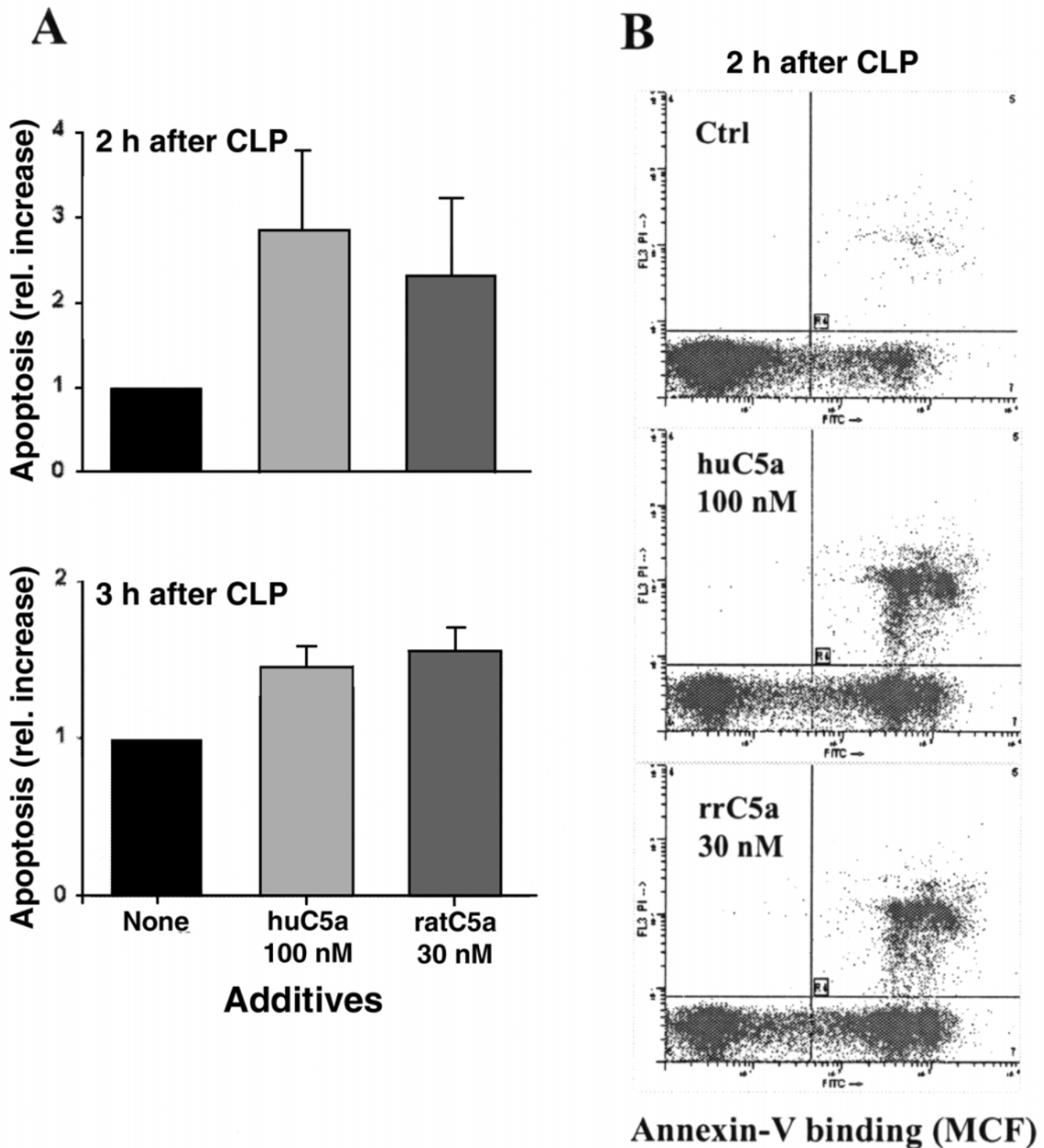
Fig. 4

Figure 4. Flow cytometry analysis for apoptosis (annexin-V binding) in rat thymocytes. A) Rat thymocytes were isolated 2 and 3 h after cecal ligation/puncture (CLP) and not otherwise stimulated or incubated with huC5a (100 nM) or rrC5a (30 nM) for 30 min at 37°C. Flow cytometry was performed after staining with annexin-V for 15 min. Data are presented as increases in apoptosis relative to the corresponding control value in each experiment. Data are from three to nine independent experiments per bar graph. **B)** Flow cytometric data from thymocytes obtained from rats 2 h after CLP and not otherwise stimulated or after exposure to huC5a or rrC5a (30 min at 37°C).
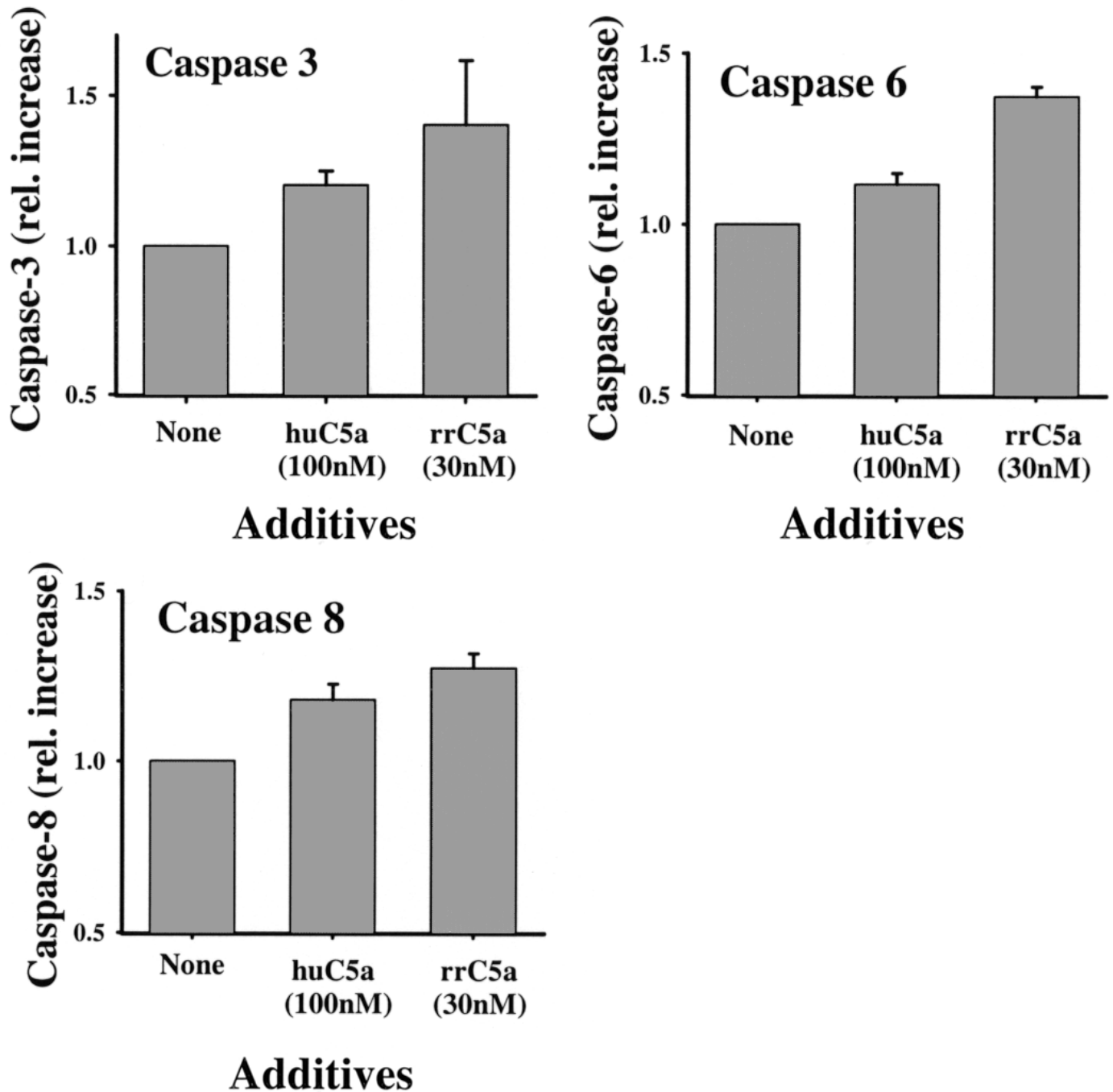
Fig. 5

Figure 5. Caspase-activation in thymocytes obtained 3 h after cecal ligation/puncture (CLP), after addition of huC5a or rrC5a. Enzymatic assays for caspases were used. Shown are relative (%) increases in caspase activities compared with the corresponding activities of nonstimulated thymocytes for each condition. The results are representative of data from two independent experiments, with each condition evaluated in quadruplicate.