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Interleukin-1β affects the macrophage recruitment and proliferation in the injured brain of 6-day-old rat

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Abstract. Six-day-old male rats received a mechanical lesion in the left cerebral hemisphere. Thereafter, a single dose of either 5, 50 or 500 units (U) of recombinant rat interleukin-1β (IL-1β) was injected into the lesion cavity. One or 2 days after the injury, the rats were injected with ^{3}H-thymidine. Brain sections were subjected to BSI-B4 lectin histochemistry and autoradiography to visualise proliferating and non-proliferating macrophages located within the region of injury. A mitogenic effect of IL-1β on macrophages was observed on day 2 in brains injected with the lowest 5 U dose of cytokine. Following administration of higher 50 U and 500 U doses, infiltration of the injured tissue by macrophages was significantly intensified on day 1. However, on day 2, dose-dependent reductions of the total number of macrophages as well as their proliferative activity were recorded. The findings suggest that the higher the initial quantity of macrophages, the sooner they disappeared from the injury site. It may therefore be hypothesised that IL-1β-induced increase in macrophage recruitment at the beginning of the inflammatory response speeded the removal of tissue debris and, therefore, accelerated healing of the injured nervous tissue.

Key words: developing brain, injury, macrophage recruitment and proliferation, IL-1β, ^{3}H-Thymidine autoradiography, BSI-B4 lectin

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INTRODUCTION

A penetrating injury to the brain breaks the blood--brain-barrier which, under normal conditions, protects nervous tissue from direct external influences (Perry et al. 1997, Martiney et al. 1998). Following immediate contact with tissue debris, blood-derived monocytes transform into macrophages and initiate their scavenger programmes. Upon entering the nervous system, macrophages become a source of cytokines controlling their own behaviour in both autocrine and/or paracrine ways (Lotan and Schwartz 1994, Ghirnikar et al. 1998). However, further infiltration of the tissue by macrophages depends also on their interaction with neurones and glia *via* a wide range of inflammatory mediators, including cytokines such as IL-1α and β, IL-6 and TNFα (Couraud 1994, Mallat et al. 1996, Silverstein et al. 1997, Sternberg 1997, Ghirnikar et al. 1998, McKeating and Andrews 1998). By releasing cytokines into the brain parenchyma, macrophages can activate resident glial cells (both astrocytes and microglia) and induce their transformation and proliferation (Balasingam et al. 1994, 1996, Rostworowski et al 1997, Ghirnikar et al. 1998). Therefore, the presence of inflammatory cells is considered as the important determinant of tissue healing and posttraumatic scar formation (Giulian et al. 1989, Arvin et al. 1996, Fitch and Silver 1997, Ghirnikar et al. 1998). Recently, we found a negative correlation between the intensity of astrocyte proliferation and macrophage recruitment and proliferation in the brain of 6-day-old rat (Janeczko et al. 1998). This attracted our attention to different possible functional relations (including antagonistic, Hailer et al. 1998) between cells invading the injured nervous tissue and those engaged in the restoration and maintenance of the blood-brain-barrier.

In the injured brain, IL-1β belongs to the cytokine set controlling initial stages of the inflammatory response (Silverstein et al. 1997, McKeating and Andrews 1998). There are multiple cellular sources of IL-1β, including macrophages themselves (McGeer and McGeer 1997, Ghirnikar et al. 1998, McKeating and Andrews 1998) and also neurones (Bartfai and Schultzberg 1993, Couraud 1994) and glia (McGeer and McGeer 1997). According to previous studies, *in vivo* administration of IL-1β evokes expression of adhesion molecules on endothelial cells which is critical for recruitment of blood phagocytes to the injured tissue (Giulian et al. 1989, Martiney et al. 1998, McKeating and Andrews 1998). Moreover, IL-1β activates (directly and/or indirectly) not only macrophages (McKeating and Andrews 1998) but also microglia (Giulian et al. 1994) and astrocytes (Giulian 1987, Balasingam et al. 1994, Rostworowski et al. 1997).

It has already been documented that cellular reactivity to injury is considerably stronger in one-week-old than in adult rat brains (Janeczko 1994, Silverstein et al. 1997). This developmental stage became a subject of special interest (Janeczko 1994, Pawlinski and Janeczko 1997b, Silverstein et al. 1997, Janeczko et al. 1998, Setkowicz and Janeczko 1998) because of vigorous regressive and progressive developmental processes occurring in the brain (Ferrer et al. 1992). While the brain develops, substantial changes in the profile of cytokine production take place (Balasingam et al. 1994, 1996, Silverstein et al. 1997). The changes can also modify the ability of nervous tissue to interact with exogenous cells invading the brain following injury (Milligan et al. 1991).

Variable effects of IL-1β on different aspects of the response to injury in the brain at different developmental stages have already been reported by several authors (Sievers et al. 1993, Balasingam et al. 1994, Giulian et al. 1994, Anthony et al. 1997). Among them, Giulian et al. (1994) showed changes in the macrophage recruitment following intracerebral administration of IL-1β, but the effects of this cytokine on macrophage prolifera-

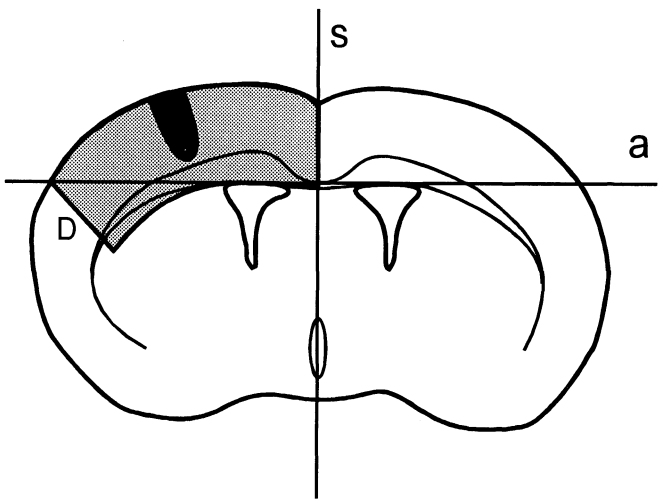

Fig. 1. Delineation of the investigated standard zone. S, axis of symmetry of the brain section; a, line perpendicular to the axis S passing through the top of the lateral ventricle; D, distance between the top of the lateral ventricle and the surface of the cerebral hemisphere. Shadowed area, the investigated zone; black area, the lesion site.

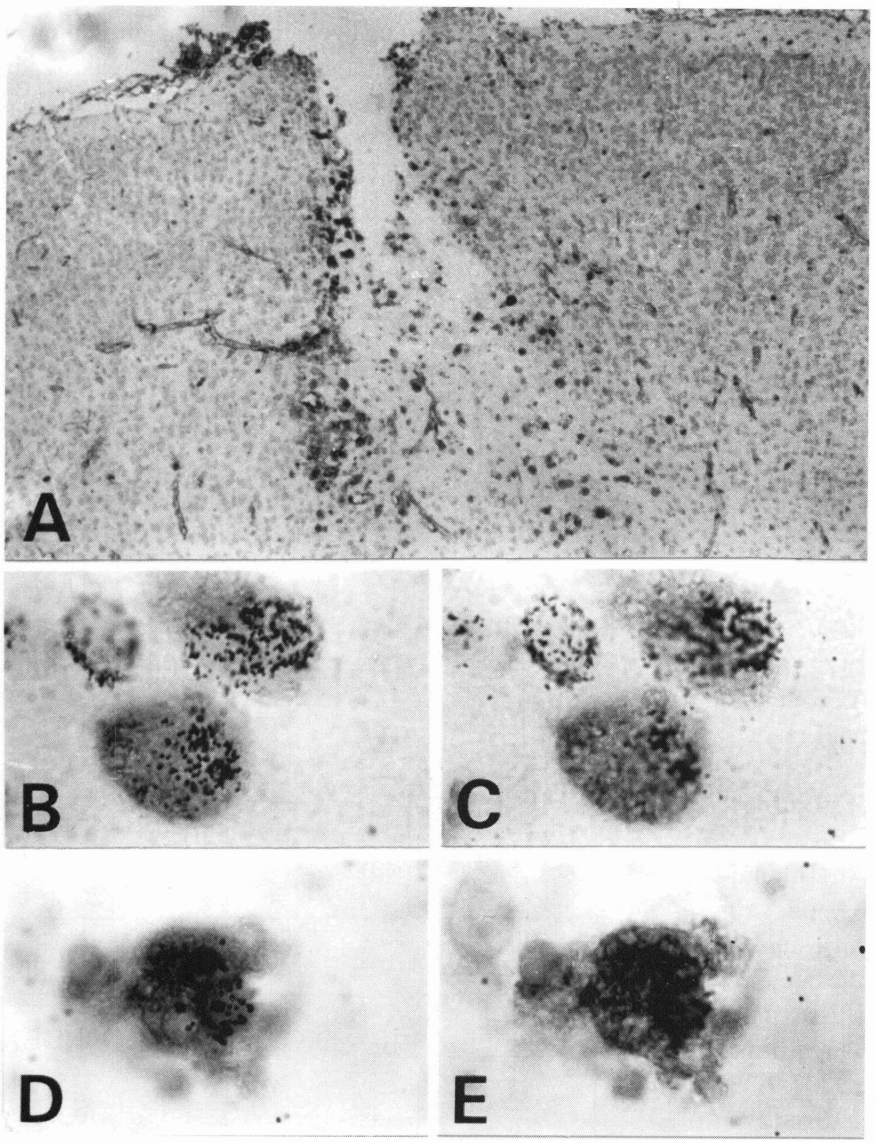

Fig. 2. A, the lesion area on day 1 after injury; x 90. Examples of lectin-positive and autoradiographically labelled macrophages are shown at higher magnification. Photographs are focused on different planes to demonstrate silver grains over cell nuclei (B, D) or macrophages labelled by them (C-E), (X1150).

tion have never been investigated in the injured brain. General studies on macrophage proliferation, even in the injured but cytokine-untreated brain, were infrequent. Moreover, the results were contradictory (Fujita et al. 1998, Janeczko et al. 1998) and, therefore, additional research was needed. The present study examined the dynamics of macrophage recruitment and proliferation in injured and IL-1β-treated rat brain.

METHODS

A more detailed description of methods used in this study was presented in our previous publication (Pawliński and Janeczko 1997a,b, Janeczko et al. 1998).

Adult Wistar rats were obtained from an animal colony at the Institute of Pediatry, Collegium Medicum, Jagiellonian University, Cracow and maintained under conditions of controlled temperature (20 ± 2°C) and illumination (12 h light/dark cycle). A solid diet (Murigran) and water were available *ad libitum*. All experimental procedures were in accordance with the guidelines instituted by the Bioethical Committee at the Jagiellonian University.

Brain lesioning

Six-day-old rats were ether-anaesthetised and lesioned under aseptic conditions. A rotating dental drill, 0.9 mm in diameter, was inserted down to the white matter underlying the cerebral cortex of the left cerebral hemisphere. The depth of penetration was limited to 1.8 mm below the skull surface by a plastic ring attached to

the drill, preventing perforation of the whole thickness of the hemispheric wall (Figs. 1 and 2A).

Intracerebral injection of interleukin-1 beta

Rat recombinant interleukin-1 beta (IL-1β, RandD) was diluted in sterile 0.9% saline. Immediately after lesioning the brain, a stainless cannula, 0.5 mm in diameter, was introduced into the lesion site perpendicularly to the skull surface and 1 µl of IL-1β solution was injected using Hammilton 2 µl syringe. The depth of penetration below the skull surface was set at 1.8 mm by a limiting plastic ring fixed to the cannula. The injection took place over a period of 2 min at a constant rate. The cannula was then slowly removed. After the skin was sutured, the animals were returned to their mothers.

Three experimental groups of animals received injections of IL-1β at dose 5, 50 or 500 units (U) in 1 µl volume. Brains of animals belonging to the control group were injected with 0.9% saline alone. Numbers of animals constituting respective groups are indicated in Fig. 3.

Autoradiography and histochemical staining

One or 2 days after the injury [methyl-^{3}H]thymidine (Institute for Research, Production and Application of Radioisotopes, Prague) was injected i.p. in a single dose of 5 µCi/g body weight. Four hours after the injection the rats were deeply anaesthetised and perfused intracardially with 0.9% NaCl followed by 4% paraformaldehyde (Sigma) in 0.1M phosphate buffer, pH 7.3. The brains were embedded in paraffin and sectioned at 7 µm in the coronal plane. Brain sections were mounted on slides and processed for specific histochemical staining with isolectin from Bandereira simplicifolia conjugated with horseradish peroxidase (BSI-B4, Sigma L 5391) dissolved in 0.5% Triton X-100-containing TBS at a concentration of 10 µg/ml. After overnight incubation in this solution the slides were washed in TBS and binding sites of lectin-HRP conjugate were visualised using 3,3'-diaminobenzidine as a chromogen. On the histochemically stained sections, autoradiographs were prepared, exposed for two weeks, then developed and counterstained with Harris haematoxylin.

Microscopic observations

Three sections from each brain at the level of the lesion were chosen for microscopic examination using a square frame containing eyepiece. At a magnification of 1,000 x, the frame delimited a 100 x 100 µm area. In each section a standard zone around the lesion was delimited (Fig. 1). The zone was then examined square by square, and all lectin-positive autoradiographically labelled or

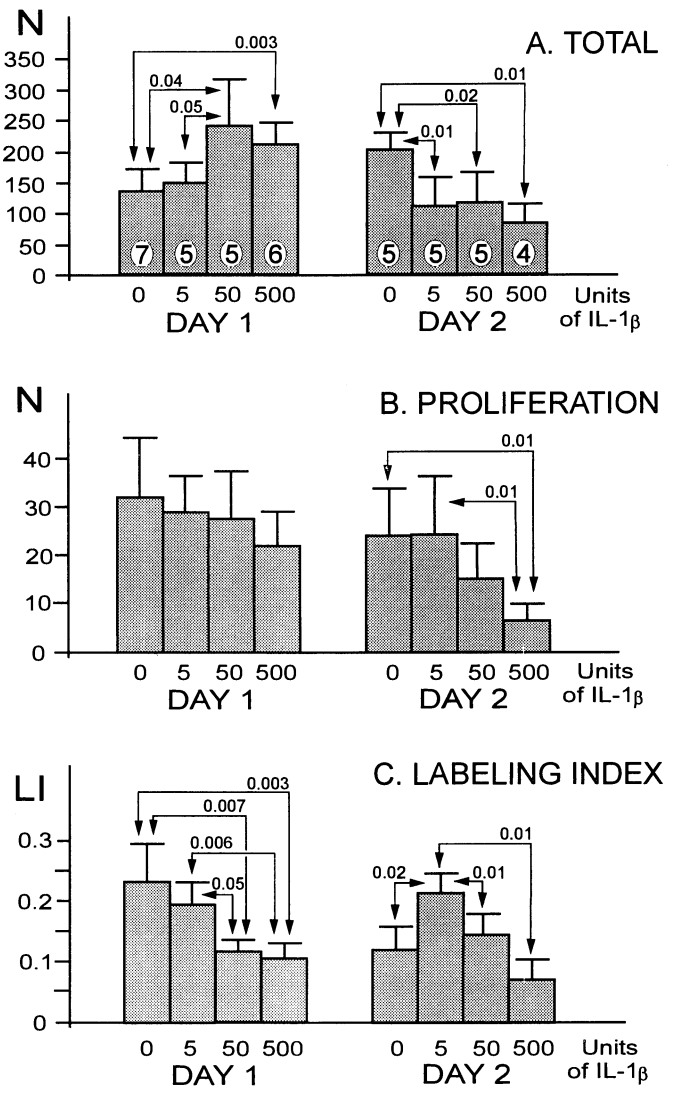

Fig. 3. Changes in the total number of macrophages (A), the number of proliferating macrophages (B) and in their labelling index (C) in relation to the dose of IL-1β injected into the lesion cavity. N, number of macrophages (± SD), LI, labelling index (± SD). The control group and groups receiving dose of 5, 50 or 500 units of IL-1β are indicated as 0, 5, 50 or 500, respectively. Decimal indexes over double-headed arrows show statistical significance of differences between different groups of animals. Numbers of animals in each group are shown in ellipsoids.

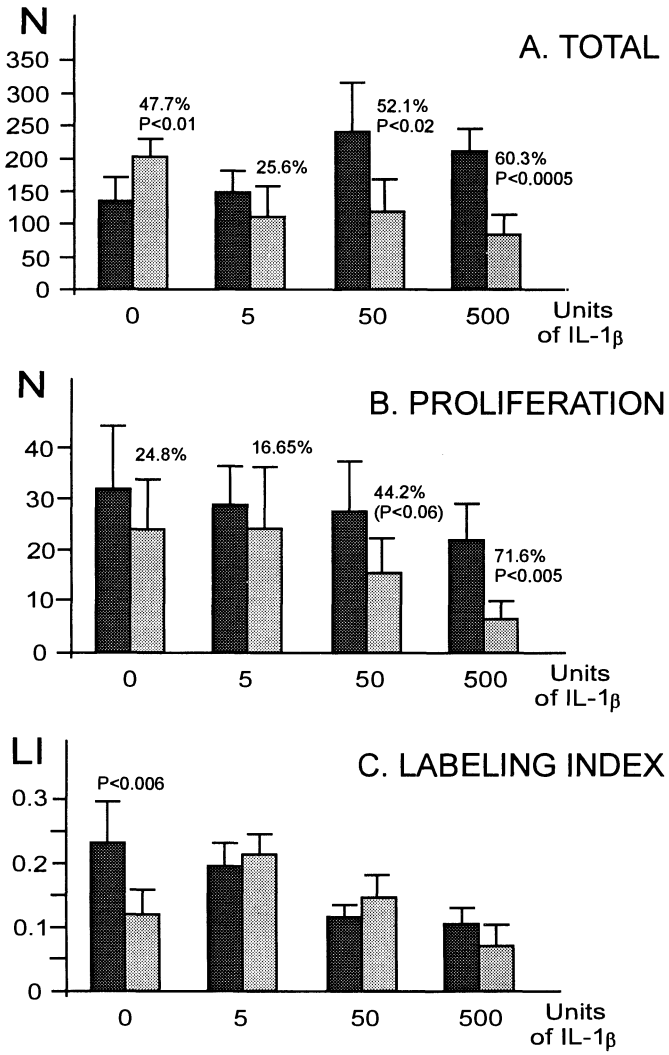

Fig. 4. Changes in the macrophage population during the period between posttraumatic days 1 and 2 in brains injected with different doses of IL-β. Dark bars - day 1 after injury, light bars - day 2 after injury. Indexes over bars show percents of increases/decreases and statistical significance of changes which occurred during the period between posttraumatic days 1 and 2. An index in brackets corresponds to a difference which was slightly below $P<0.05$ standard of statistical significance. For further explanations see Fig. 3.

unlabelled macrophages were recorded separately. The macrophages were considered as autoradiographically-labelled when they showed at least 6 silver grains over their nuclei (Fig. 2). The autoradiographic labelling was an indicator of proliferative activity of the cells.

Microscopical observations were carried out without knowledge of the previous treatment of the rats. For each animal group, average numbers of proliferating and non-proliferating macrophages were calculated. An index of proliferative activity (labelling index) was also determined as the percent of proliferating macrophages in relation to their total number.

Statistical significance of differences between all the experimental and control groups was estimated using the Mann-Whitney test.

RESULTS

Within the lesion cavity and in the close vicinity to its margins, numerous macrophages showing very strong lectin histochemical staining were found. They could also be seen in the white matter over the top of the lateral ventricle. Large, round or oval cell bodies were frequently filled with tissue debris (Fig. 2). Some of them displayed proliferative activity as indicated by silver grains over their nuclei.

Compared to controls, brains injected with IL-1β demonstrated changes both in the total number of macrophages and in the intensity of their proliferation (Fig. 3). Since the range of those changes varied in relation to different doses of the cytokine, we tried to assess the proliferative activity of macrophages by calculating an autoradiographical labelling index, i.e. the ratio between the number of autoradiographically labelled macrophages and their total number.

On day 1 after injury, the minimal 5 U dose of IL-1β had no effect but application of intermediate (50 U) and maximal (500 U) doses caused statistically significant but similar increases in the total number of macrophages ($P<0.04$ and $P<0.003$, respectively, Fig. 3A). However, the number of proliferating macrophages did not change significantly except for a slight decline related to increasing doses of injected IL-1β (Fig. 3B). As a result of the above changes, labelling indexes characterising brains injected with intermediate and maximal doses were strongly reduced ($P<0.007$ and $P<0.003$, respectively, Fig. 3C).

Compared with those on day 1, different changes took place on day 2 after injury. Similar, statistically significant but not dose-dependent, reductions in the total number of macrophages could be seen in each experimental group (Fig. 3A). The average number of proliferating macrophages was inversely proportional to the increasing dose of injected IL-1β (Fig. 3B). The changes caused corresponding statistically significant variations of the labelling index (Fig. 3C). For the minimal dose, the index was significantly higher than that in control brains ($P<0.02$). After injection of intermediate and maximal doses, the index became gradually lower ($P<0.01$ for

each) in relation to the effect of the minimal dose but not to the control group (Fig. 3C).

The most interesting differences were seen in the dynamics of the macrophage infiltration and proliferation (Fig. 4). In control brains, a 47.7% increase in the total number of macrophages occurred between the 1st and 2nd posttraumatic days ($P<0.01$). However, during the same period, in brains injected with three increasing amounts of IL-1β, a systematic regression of the total number of macrophages was recorded at levels of 25.6% (statistically insignificant), 52.1% ($P<0.02$) and 60.3% ($P<0.0005$), respectively (Fig. 4A). The number of proliferating macrophages underwent very similar dose-dependent declines (Fig. 4B). In consequence, in each group of IL-1β-injected animals, the labelling index remained at almost the same level even though the real values displayed dose-dependent variations (Fig. 4C). By contrast, in control brains, the labelling index showed a significant decrease during the period between days 1 and 2 after injury ($P<0.006$, Fig. 4C).

DISCUSSION

Microscopic observations revealed macrophages dispersed mostly within the lesion cavity and in the tissue adjacent to its margins. Using lectin histochemistry, however, it was impossible to distinguish macrophages derived from circulating mononuclear phagocytes from those originating from resident microglia (Thomas 1992). The additional presence of macrophages in the white matter over the lateral ventricle was typical of brains of one-week-old rats (Ferrer et al. 1992). That developmental stage became a subject of special interest in several studies, since it is characterised by highly active progressive and regressive processes such as gliogenesis and programmed cell death (Ferrer at al. 1992, Silverstein et al. 1997). The processes include increased production of multiple factors and expression of their receptors which may significantly intensify tissue reactivity to injury (Janeczko 1994, Lawson and Perry 1995, Silverstein et al. 1997)

In the present study, the reactive behaviour of macrophages was displayed by changes in their total number (to indicate the intensity of recruitment into the injured brain) and in the number of their mitoses. Days 1 and 2 after injury, which were chosen for examination, represented the period when the two parameters reached relatively high levels. In the control group, macrophage proliferation peaked on day 2 but became close to the control level on day 4 (data not shown).

Because of considerable variations of the two parameters, the labelling index was calculated to assess more precisely the proliferative activity of the cells. The index characterises a cell population growing exclusively by mitotic activity. When calculated for macrophages present in the injured brain, it could be modified not only by acceleration the cells own proliferation but also by an influx of exogenous phagocytes. Therefore, the labelling index was always compared with corresponding changes in the total number of macrophages.

The present study revealed some important effects of IL-1β on the reactive behaviour of macrophages. When compared to the control brains, the total number of macrophages increased on day 1 after injury and decreased on day 2. However, the total number of macrophages was not related to the dose of cytokine. On the other hand, the number of proliferating macrophages on day 2 did showed a dose-dependent decline.

Interrelations between total numbers of macrophages and their mitoses were reflected by changes in the labelling index. On day 1 after injury the index was inversely related to the increased amount of injected cytokine. That might result from a suppressive effect of higher doses of IL-1β on proliferating macrophages which appeared at the injury site in considerably increased quantities. It is noteworthy, however, that the macrophage population, which was considerably reduced on day 2 following administration of the lowest 5 U dose of IL-1β, displayed the highest number of mitotic cells. That was shown by an 81.4% elevation of the macrophage labelling index in relation to control brains, which was the only positive effect of IL-1β on macrophage proliferation. Further increases in the amount of cytokine led to significant dose-dependent decreases in the number of mitotic macrophages. It was accompanied by a strictly proportional reduction of the labeling index, related, however, to the effect of the minimal dose. This was additional evidence of a decreasing response of macrophage proliferation to an increasing dose of cytokine.

At present, it is difficult to propose a mechanism underlying the observed changes, since they might be related to at least two indistinguishable exo- and endogenous cell populations. The populations might present different type-specific patterns of reactivity even though they were intermingled at the injury site.

Another interesting result of the study is that the total number of macrophages as well as their mitoses, which

occurred during the period between the 1st and 2nd post-traumatic days were closely related to the injected dose of IL-1β. Therefore, it became obvious that, for an adequate assessment of the cytokine effect, the number of macrophages recorded on day 2 after injury needed to be compared not only to their number in control brains (Fig. 3) but also to their number in cytokine-injected brains on day 1 (Fig. 4).

The above observations suggest the following cytokine-induced scenario: the two higher doses (50 U and 500 U) of IL-1β (but not 5 U) increased the macrophage population within the injured area on day 1 after injury (Fig. 3A). Thus, the macrophage recruitment from circulating mononuclear phagocytes and/or from resident microglia proved, in part at least, to be dose-dependent. A considerable decline in the total number of macrophages during the next 24 h was strictly proportional to the amount of injected cytokine (Fig. 4A). It might, therefore, be supposed that an increase in the initial number of macrophages observed in the IL-1β-injected brains led to a higher rate of debris removal (Giulian 1987). The macrophage proliferation demonstrated a similar temporal pattern of changes. The efficiency of removal might also be higher since elevated concentrations of IL-1β could stimulate a specific activity of phagocytes (Moxey-Mims et al. 1991) and production of other cytokines like IL-6, TNFα and GM-CSF, increasing further recruitment of macrophages and/or the rate of phagocytosis (Martiney et al. 1998, McKeating and Andrews 1998). Therefore, because of the initial cytokine-induced reinforcement of the macrophage activity, phagocytosis could terminate earlier. This might lead to a quicker disappearance of macrophages from the lesion site indicating a general decline in the intensity of reactive processes. To evaluate this hypothesis, processes underlying the observed phenomena, especially those determining the disappearance of macrophages and their final destination, need further detailed examination.

In comparison to IL-1β-injected brains, the control brains showed opposite changes: the total number of macrophages on day 2 after injury was significantly higher than that on day 1. That may suggest that tissue clearance was still developing so the process should last relatively longer than in IL-1β-injected brains. It might, therefore, be concluded that, when applied at a low dose, IL-1β was mitogenic for macrophages located at the injury site. However, at higher doses, the cytokine intensified the macrophage recruitment into the injured brain, while simultaneously suppressing the proliferative activity. The IL-1β-induced increase of the macrophage population occurring at the initial stage of inflammatory response might, therefore, lead to a dose-dependent acceleration of nervous tissue healing.

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