Clin Exp Immunol 1998; **111**:198–204

Effect of annexin-1 on experimental autoimmune encephalomyelitis (EAE) in the rat

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(Accepted for publication 7 October 1997)

SUMMARY

Annexin-1, a calcium-dependent phospholipid binding protein, has been shown to act as an endogenous central neuroprotectant, notably against cerebral ischaemic damage. In the present study we extend these findings to an animal model of multiple sclerosis, EAE, and report that endogenous annexin-1 is expressed in $ED1^+$ macrophages and resident astrocytes localized within the lesions in the central nervous system (CNS). Intracerebroventricular (icv) administration of an NH_2-terminal fragment spanning amino acids 1–188 of annexin-1 after the onset of the clinical symptoms significantly reduced both the neurological severity as well as weight loss of mild EAE. Immunoneutralization of endogenous brain annexin-1 failed to exacerbate the clinical features of EAE. Thus, although the role of endogenous annexin-1 in the pathogenesis of EAE remains to be determined, our findings suggest that annexin-1 may be of therapeutic benefit to the treatment of multiple sclerosis.

Keywords annexin-1 lipocortin-1 experimental allergic encephalomyelitis central nervous system astrocytes macrophages

INTRODUCTION

EAE represents an animal model for demyelinating diseases of the central nervous system (CNS) like multiple sclerosis (MS). EAE can be induced in Lewis rats after immunization with homogenized myelin, or myelin basic protein (MBP) in Freund's complete adjuvant (FCA). About 9–10 days after immunization, encephalitogenic T cells and impressive numbers of macrophages infiltrate the CNS, forming large perivascular inflammatory cuffs [1]. Oedema and demyelination are subsequently responsible for the neurological deficits, which include paresis and paralysis of the tail and the hind limbs. EAE is also accompanied by an impressive loss in body weight [1].

Corticosteroid treatment has proved beneficial in both MS and EAE [2,3]. Elevated plasma concentrations of glucocorticoids are also necessary for the spontaneous recovery from clinical EAE [4]. How glucocorticoids suppress EAE is not clear, but it may be mediated in part by the local reduction of cellular infiltration and inflammatory reactions in the CNS. Glucocorticoids inhibit the production of proinflammatory molecules such as IL-1β, IL-6, tumour necrosis factor-alpha (TNF-α) and eicosanoids, but induce the expression of annexin-1 [5–8]. Annexin-1, also known as lipocortin-1, is a 37-kD member of a family of structurally related Ca^{2+} and phospholipid binding proteins [5–9]. Annexin-1 has evoked interest as a glucocorticoid-inducible protein that can suppress the production of eicosanoids by inhibition of phospholipase A_2 (PLA_2) and arachidonic acid release [10–17]. The protein is expressed constitutively in the immune system in macrophages and polymorphonuclear cells (PMN), and also in the CNS, where it is located in neurones, astrocytes and ependymal cells lining the cerebral ventricles [18,19]. Furthermore, annexin-1 is present at nanomolar concentrations in human serum [20]. Glucocorticoid analogues can up-regulate the constitutive expression of annexin-1 in some cell types, and glucocorticoid receptor antagonists or adrenalectomy reduce constitutive expression of annexin-1 also in site- and cell type-dependent ways [7,19,21]. Annexin-1 is up-regulated in the brains of MS patients as well as of rats with EAE, possibly due to the rise in plasma cortisol and corticosterone levels during disease [4,22,23].

Functional studies using recombinant full length annexin-1, an NH_2-terminal fragment spanning amino acids 1–188 of annexin-1 (annexin-1 (1–188)) or amino acids 2–26 (annexin-1 (2–26)), and neutralizing antibodies raised against annexin-1 (1–188) have revealed involvement of annexin-1 in inflammation and neurodegeneration. Annexin-1 (1–188) reduces febrile responses to

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cytokines [24,25]. Also, neurodegeneration caused by cerebral ischaemia- or N-methyl-D-aspartate (NMDA) receptor-mediated tissue damage in the rat is reduced by annexin-1 (1–188) [26,27]. In addition, anti-inflammatory actions of exogenous full length annexin-1 have been demonstrated in an animal model of lung perfusion and a model of paw oedema [28,29], and migration of PMN to an inflamed peritoneal cavity is also greatly reduced by annexin-1 (2–26) as well as by annexin-1 (1–188) [30]. Anti-inflammatory effects of dexamethasone, like suppression of oedema and inhibition of the production of eicosanoids, TNF-α and nitric oxide (NO), can be reversed by annexin-1 antiserum [31–33]. Together with the induction of annexin-1 by dexamethasone, these findings support the hypothesis that annexin-1 can act as key mediator of the anti-inflammatory actions of glucocorticoids [31–33].

To gain insight into annexin-1 production and function in the CNS during EAE we investigated the spatial and temporal pattern of immunoreactive annexin-1 expression in the CNS of rats during EAE. Furthermore, we studied the effects of annexin-1 (1–188) on the clinical manifestation of EAE. Finally, we analysed the role of endogenous annexin-1 in the clinical expression of EAE using neutralizing antibodies.

Here we report the novel findings that: (i) annexin-1 is induced in the CNS during clinical manifestation of EAE in ED1$^+$ macrophages as well as in reactive astrocytes in the lesions; and (ii) administration of annexin-1 (1–188) protected against clinical manifestations of mild EAE.

MATERIALS AND METHODS

Animals

Male Lewis rats were obtained from the Zentral Institut für Verzuchstierzucht (Hannover, Germany) or the Broekman Instituut (Someren, The Netherlands), and maintained under standard laboratory conditions. Water and food were available *ad libitum*. The animals weighed $\approx$200 g at the start of experiment.

Reagents

Annexin-1 (1–188) (amino acid sequence 1–188 of the NH_2-terminal of human annexin-1) was kindly donated by Dr F. Carey (Zeneca Pharmaceuticals, Macclesfield, UK) [24]. Polyclonal anti-annexin-1 antibodies were raised in a rabbit against the human recombinant annexin-1 (1–188) [24]. A purified mouse MoAb to annexin-1 which had been raised against full-length bovine annexin-1 was obtained commercially from Zymed (San Francisco, CA) (clone ZO13). Mouse MoAb ED1, recognizing all rat macrophages, was produced in our laboratory and used after protein A purification [34].

Experimental allergic encephalomyelitis

Acute EAE was induced under ether anaesthesia by a single subcutaneous injection in the hind foot pad of 60 μl of the following emulsions: guinea pig spinal cord (GSC) homogenate (1 g GSC/ml saline; experiments 1 and 2), or 25 μg of MBP in saline (experiment 3), emulsified in an equal volume of FCA, to which 10 mg *Mycobacterium tuberculosis* H37 RA (Difco Labs, Detroit, MI) per ml of FCA were added. The rats were weighed and investigated daily for the development of neurological symptoms. Clinical signs were scored on a scale ranging from 1 to 5 as described previously [1].

Immunocytochemistry

For annexin-1 (1–188) and ED1 staining, rats were perfused transcardially with Ringer solution pH 6·9 followed by 4% paraformaldehyde (PFA) in 0·1 M phosphate buffer. MoAb ED1 is specific for all rat macrophages [34]. The specificity of the polyclonal antibody raised against annexin-1 (1–188) has been described earlier [19]. Preabsorption of the antibody with 10^{-6} M annexin-1 (1–188) resulted in absence of annexin-1 staining in the rat brain [19]. Furthermore, stainings in which the first antibody was omitted resulted in absence of annexin-1 immunoreactivity in sections of the CNS of rats with full-blown clinical EAE (data not shown). Brains were dissected and post-fixed for 24 h in 4% PFA. Cryostat sections of 10 μm were double-stained with anti-annexin-1 (1–188) polyclonal rabbit antibody and ED1 diluted 1:100 and 1:300, respectively, in 0·05 M Tris-buffered saline pH 7·6 to which 2·5% BSA was added (TBS–BSA) overnight at 4°C. Primary antibodies were visualized using FITC or TRITC-labelled secondary antibodies (Jackson ImmunoResearch Labs, West Grove, PA). To prepare 1-μm sections, vibratome sections of 50 μm were incubated with anti-annexin-1 (1–188) polyclonal rabbit antibody diluted 1:100 in TBS–BSA for 48–72 h at 4°C. After rinsing, sections were incubated with biotin-conjugated anti-rabbit antibodies (Dakopatts, Tilburg, The Netherlands), which were subsequently visualized using peroxidase-labelled avidin–biotin complex (ABC; Vector Labs, Burlingame, CA) and 3,3′ diaminobenzidine-tetra-hydrochloride (DAB; Sigma, St Louis, MO), respectively. Annexin-1-stained sections were embedded in epon as described previously [35]. Selected areas from the 50-μm epon-embedded sections were cut out and mounted on prepared epoxy beams and semi-thin 1-μm sections were prepared and counterstained with toluidine blue.

Intracerebroventricular injections

For intracerebroventricular (icv) treatment administration of saline, annexin-1 (1–188) or antibodies, a guide cannula (internal diameter 0·58 mm, external diameter 0·96 mm) was placed stereotactically into the lateral ventricle as described earlier at least 7 days before the induction of EAE [36]. Treatment solutions were injected icv at a rate of 2 μl/min using a stainless steel injector (external diameter 0·5 mm) and a microinjection pump (Harvard Apparatus, South Natick, MA).

Experimental design

Cellular localization of annexin-1 and ED1. Brains were taken 5, 12 (fluorescence double-labelling) or 15 days (semi-thin sections) after EAE induction.

EAE icv treatment studies. In experiment 1, annexin-1 (1–188) (0·48 μg/μl saline, $n=5$) or saline ($n=5$) was injected (5 μl, icv) once daily before noon at 8–12 days after induction of EAE.

In experiment 2, polyclonal antiserum to annexin-1 (1–188) ($n=6$) or saline ($n=6$) was injected (5 μl, icv) once before noon at 9 days after EAE induction.

In experiment 3, annexin-1 (1–188) (0·48 μg/μl, $n=6$), a MoAb to annexin-1 (1 μg/μl, $n=5$) or saline ($n=7$) was injected repeatedly (5 μl, icv) once daily before noon at 9–13 days after EAE induction. This experiment included a non-cannulated control group ($n=7$) that received no treatment after EAE induction.

Statistical analysis

Effects of annexin-1 (1–188) treatment on EAE were evaluated statistically by analysing the incidence of clinical disease of EAE

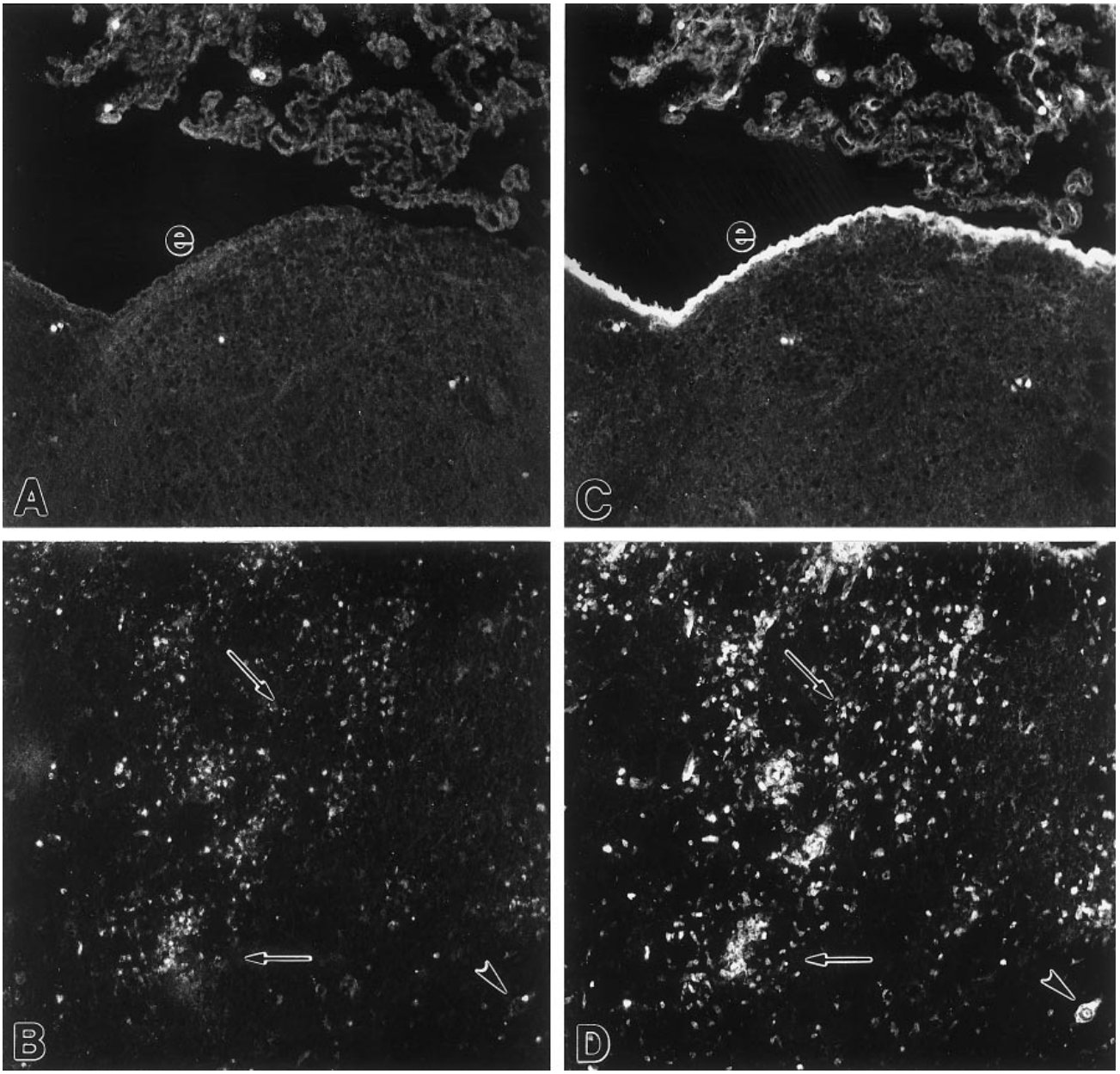

Fig. 1. Microphotograph of a double-staining of ED1 (A,B) and annexin-1 (C,D) immunoreactivity in brains of rats with EAE. (A,C) Coronal section through the medulla oblongata 5 days after the induction of EAE. (B,D) Coronal section through the diencephalon 12 days after induction of EAE. e, Ependymal cells; arrowhead, neurone; arrows point at examples of annexin-1-positive cells that are not ED1$^+$. Note that most ED1$^+$ cells exhibit annexin-1 immunoreactivity. Mag. $\times$ 100.

using χ^2 test. The duration of clinical disease of EAE was analysed using Student's *t*-test for unmatched data, and weight loss during EAE was analysed using the Mann–Whitney *U*-test. Statistical significance is defined as $P < 0{\cdot}05$.

RESULTS

Annexin-1 immunoreactivity in the CNS of rats with EAE

In non-immunized healthy rats, no ED1 reactivity was seen in the CNS (not shown) and annexin-1 immunoreactivity was confined to ependymal cells and tanycytes, as described earlier [19]. Five days after induction of EAE, annexin-1 immunoreactivity was confined mainly to ependymal cells (Fig. 1C). At this stage of EAE, there were no detectable ED1$^+$ macrophages in the CNS (Fig. 1A). Twelve days after the induction of EAE, when the clinical manifestation of the disease is maximal, many ED1$^+$ macrophages (Fig. 1B) and annexin-1-immunoreactive cells (Fig. 1D) were found in the lesions. Double-staining demonstrated that many, but not all, annexin-1-immunoreactive cells were in fact ED1$^+$ macrophages (Fig. 1B,D). Brain sections of inflammatory lesions 15 days after the induction of EAE revealed that, in addition to macrophages, astrocytes also displayed intense annexin-1 immunoreactivity. These were found predominantly around small vessels in the lesions (Fig. 2).

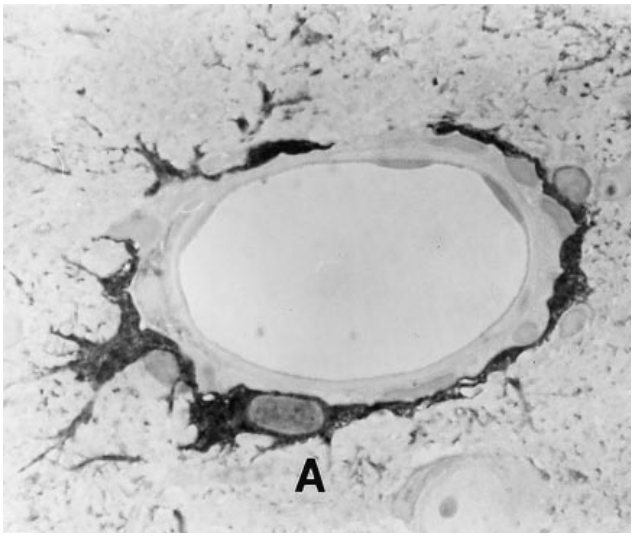

Fig. 2. Microphotograph of annexin-1 immunoreactivity in a 1-μm section of a lesion in the brain 15 days after the induction of EAE. A, Astrocyte. Mag. $\times$400.

Experiment 1: effect of repeated icv administration of annexin-1 (1–188) on EAE

Saline-treated rats first developed neurological symptoms 9 days after the induction of EAE (Fig. 3). Incidence of clinical symptoms was 100% (5/5) in saline-treated rats and varied between paralysis of the tail (5/5) and paralysis of the hind limbs (2/5). Repeated daily icv administration of annexin-1 (1–188), starting 1 day before the anticipated onset of the clinical manifestation of the disease (i.e. 8–12 days after induction of EAE) markedly reduced the severity of EAE compared with saline-treated rats ($P < 0{\cdot}02$, χ^2 test, Fig. 3). Only three out of five animals treated with annexin-1 (1–188) developed clinical EAE, and severity of disease was confined to partial or complete paralysis of the tail. Duration of clinical disease was $5{\cdot}6 \pm 2{\cdot}3$ days in the control group and $2{\cdot}3 \pm 1{\cdot}9$ days in the annexin-1 (1–188)-treated group ($P < 0{\cdot}04$, Student's *t*-test). Weight loss, which accompanies the clinical phase of EAE, was significantly reduced in the annexin-1 (1–188)-treated group compared with the

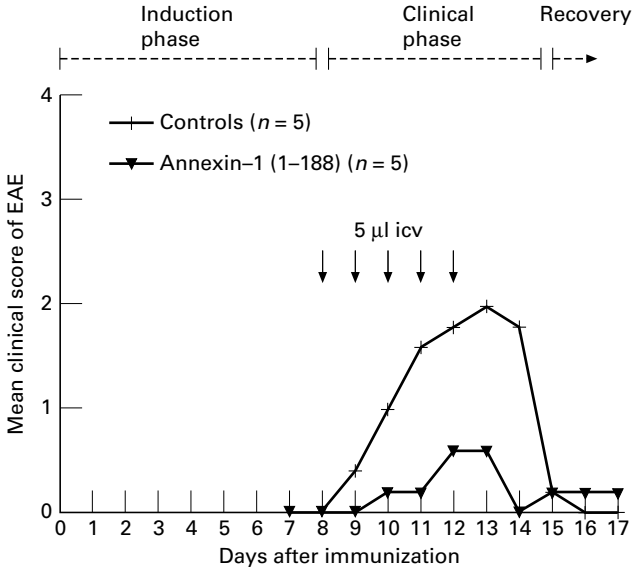

Fig. 3. Effect of repeated intracerebroventricular (icv) administration of annexin-1 (1–188) on clinical signs of EAE (experiment 1). EAE was induced by injecting guinea pig spinal cord (GSC) homogenate. Rats were given once daily icv injections (arrows) of annexin-1 (1–188) ($2{\cdot}15\,\mu$g in $5\,\mu$l in $2{\cdot}5$ min, $n = 5$) or saline (controls, $5\,\mu$l in $2{\cdot}5$ min, $n = 5$) at 8–12 days after the induction of EAE. Treatment with annexin-1 (1–188) reduced the clinical severity of EAE ($P < 0{\cdot}02$, χ^2 test) as well as duration of EAE ($P < 0{\cdot}04$, Student's *t*-test).

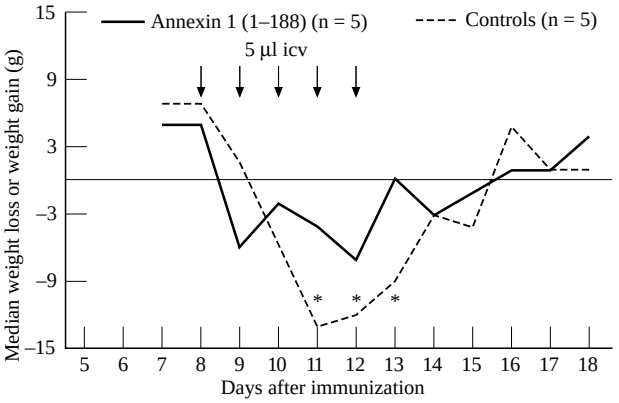

Fig. 4. Effect of repeated intracerebroventricular (icv) administration of annexin-1 (1–188) on body weight during EAE (experiment 1). The median of daily weight loss or weight gain per group is given in grams. EAE was induced by injecting guinea pig spinal cord (GSC) homogenate. Rats were given daily icv injections (arrows) of annexin-1 (1–188) ($2{\cdot}15\,\mu$g in $5\,\mu$l in $2{\cdot}5$ min, $n = 5$) or saline (controls, $5\,\mu$l in $2{\cdot}5$ min, $n = 5$) at 8–12 days after induction of EAE. Treatment with annexin-1 (1–188) reduced weight loss during clinical disease of EAE ($P < 0{\cdot}01$ at day 11, $P < 0{\cdot}04$ at day 12, and $P < 0{\cdot}05$ at day 13 after induction of EAE, Mann–Whitney *U*-test).

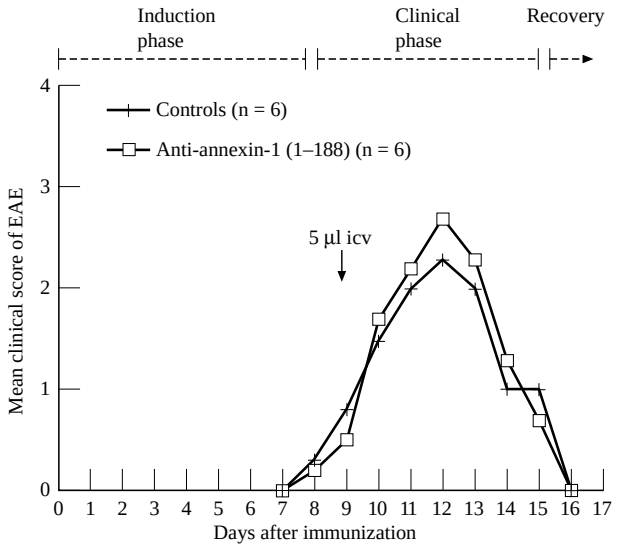

Fig. 5. Effect of a single intracerebroventricular (icv) injection of polyclonal anti-annexin-1 (1–188) on EAE (experiment 2). EAE was induced by subcutaneous injection of guinea pig spinal cord (GSC) homogenate. Rats were treated once icv at day 9 (arrow) with polyclonal antibodies against annexin-1 ($5\,\mu$l in $2{\cdot}5$ min, $n = 6$) or saline (controls, $5\,\mu$l in $2{\cdot}5$ min, $n = 6$).

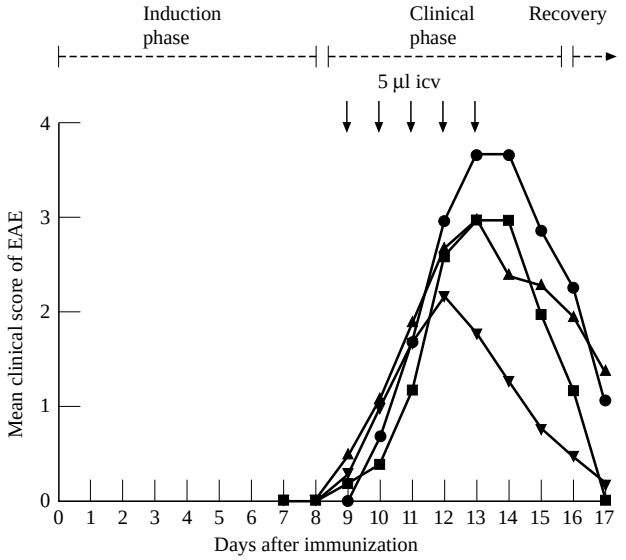

Fig. 6. Effects of repeated intracerebroventricular (icv) injections of annexin-1 (1–188) or anti-annexin-1 MoAbs on EAE (experiment 3). EAE was induced by subcutaneous injection of myelin basic protein (MBP). Non-cannulated controls (●): rats that did not receive an icv cannula and that were not treated after induction of EAE ($n=7$). Other groups of rats received icv cannulas and were treated once daily icv with annexin-1 (1–188) (2·15 μg in 5 μl in 2·5 min, $n=6$; ▼), a MoAb against annexin-1 (5 μg in 5 μl in 2·5 min, $n=5$; ■) or saline (vehicle controls, 5 μl in 2·5 min, $n=7$; ▲), at 9–13 days after induction of EAE. Note that the neurological symptoms of EAE were most severe in intact non-cannulated rats.

control treated group 11–13 days after induction of EAE ($P<0·01$ at day 11, $P<0·04$ at day 12 and $P<0·05$ at day 13; Mann–Whitney *U*-test, Fig. 4).

Experiment 2: effect of a single icv administration of antibodies to annexin-1 (1–188) on EAE

Clinical signs of EAE varied from paralysis of the tail to paralysis of the hind limbs in rats treated once either with saline or with anti-annexin-1 (1–188) antiserum at day 9 after induction of EAE (Fig. 5). No differences were observed in the severity of neurological disease or in weight loss during EAE between saline-treated and antibody-treated rats (data not shown).

Experiment 3: effect of repeated icv administration of annexin-1 (1–188) or a MoAb to annexin-1 on EAE

As illustrated in Fig. 6, clinical disease of EAE was more severe in control rats without an icv cannula compared with cannulated animals. Non-cannulated rats all developed paralysis of the complete lower part of the body (7/7, score 4). Based on the incidence of score 4 the clinical EAE was more severe in these non-cannulated rats compared with cannulated saline-treated rats (3/7 rats had score 4), anti-annexin-1 (1–188)-treated rats (2/5 had score 4, both $P<0·018$, χ^2 test) as wells as annexin-1 (1–188)-treated rats (2/6 had score 4, $P<0·007$, χ^2) (Fig. 6). Icv injections with annexin-1 (1–188) at 9–13 days after EAE induction did not reduce severity of clinical EAE ($P<0·18$, Student's *t*-test). Anti-annexin-1 MoAbs injected at days 9–13 after EAE induction had no effect on clinical expression of EAE compared with control treated rats.

DISCUSSION

This is the first study to report suppressive effects *in vivo* of annexin-1 (1–188) in an experimental autoimmune model. Repeated icv administration of recombinant annexin-1 (1–188) during the first part of the clinical phase of EAE markedly suppressed the clinical manifestation of the disease. In addition, we demonstrate the appearance of annexin-1 immunoreactivity in ED-1$^+$ macrophages and astrocytes in the CNS during the clinical and early recovery phase of EAE.

Annexin-1 has powerful immunosuppressant properties, as shown by *in vitro* and *in vivo* studies [26–30]. The immunosuppressive effects of annexin-1 may relate to its ability to inhibit PLA_2 [15,16], and the consequent reduction of proinflammatory eicosanoid production. Eicosanoids include prostaglandins, leukotrienes and thromboxanes, which can act on the vasculature to cause chemoattraction of immunocompetent cells, to increase blood flow and to enhance vascular permeability [37]. Indeed, blockade of eicosanoid synthesis suppresses EAE, emphasizing their proinflammatory role in EAE [38]. In addition, annexin-1 may interfere with other, non-eicosanoid-related, proinflammatory processes. This is indicated by the observation that inhibition of leucocyte migration to inflammatory sites by annexin-1 (2–26) could not be mimicked by selective eicosanoid blockers [30]. Recently, it has been demonstrated that annexin-1 (1–188) is also involved in the dexamethasone-induced suppression of TNF-α release from peripheral blood mononuclear cells [32]. Because a role of TNF-α in the clinical expression of EAE has been demonstrated [39], suppression of TNF-α release by annexin-1 in EAE can be expected to reduce clinical severity. In addition, it has been demonstrated that annexin-1 suppresses the clonal expansion of MBP-specific T cells [40]. Thus, icv-injected annexin-1 (1–188) may have suppressed EAE by interfering with the antigen-specific drive of the immune response in the CNS during EAE by inhibiting T cell expansion in the perivascular lesions on the one hand, and on the other hand by reduction of the release of proinflammatory mediators like eicosanoids and TNF-α by activated macrophages around the inflamed blood vessels.

Although more severe EAE tended to be reduced by annexin-1 (1–188) treatment, it was not statistically significant. Possibly, severe EAE induces higher levels of endogenous annexin-1 compared with mild EAE. In such a situation additional annexin-1 (1–188) may have relatively small effects. Alternatively, the proinflammatory drive during severe EAE may be too strong to be arrested by annexin-1 (1–188). Interestingly, chronic implantation of an icv cannula suppressed clinical severity of EAE significantly. The mechanisms underlying this suppression are not clear, but may involve neuroplasticity within the hypothalamic–pituitary–adrenal (HPA) axis induced by brain surgery, which can induce hyperresponsivitity of the axis and increased corticosterone responses to various stimuli [41,42].

Administration of anti-annexin-1 antisera did not affect the clinical course of EAE. The polyclonal annexin-1 (1–188) antibody used in our study can bind and neutralize the annexin-1 fragment [24], and icv administration of this antibody reduces tissue damage after cerebral ischaemia and inhibits pyrogenic effects of cytokines at the same dose as used in the present study [24,27]. In addition, the annexin-1 MoAbs have been shown to

block the activity of rat annexin-1 *in vitro*, although this has not been tested *in vivo* [43]. It should be noted that the effects of annexin-1 antibodies on ischaemia and fever were studied over a much shorter time period and therefore might be more effective compared with the present study.

Glucocorticoids suppress EAE [3], and activation of the HPA axis during EAE is necessary for recovery [4]. Annexin-1 immunoreactivity has been demonstrated in neurones located in areas involved in the control of the HPA axis [19,21] and both *in vitro* and *in vivo* studies suggest that annexin-1 is a mediator of the negative feedback actions of glucocorticoids after inflammatory mediator-induced activation of the HPA axis [43–45]. This implies that icv injection of annexin-1 (1–188) could mimic the negative feedback effects of glucocorticoids and therefore inhibit activation of the HPA axis, which would counteract anti-inflammatory effects of the annexin-1 treatment [4]. Conversely, anti-annexin-1 antibodies can suppress the negative feedback effects of glucocorticoids which would lead to sustained activation of the HPA axis. This phenomenon could have contributed to the absence of effects of anti-annexin-1 antibodies on the clinical course of EAE in this study.

In the healthy rat brain annexin-1 immunoreactivity is prominent in ependymal cells, choroid epithelial cells and a particular population of astrocytes called 'tanycytes', and is present in distinct neural cell populations [19]. Several studies describe up-regulation of annexin-1 in the CNS after brain damage or inflammation [22,23,46,47]. This is mainly restricted to reactive astrocytes and infiltrating macrophages after haemorrhage, embolic or traumatic infarctions in human CNS and in Alzheimer's disease, as well as in kainate-induced lesions in the CNS of rats [46,47]. The results of our study confirm earlier findings on the increase of annexin-1 immunoreactivity in the CNS during EAE [22], and identify ED-1^+ macrophages and astrocytes as major production sites of annexin-1 in this experimental disease. The appearance of annexin-1 immunoreactivity in perivascular astrocytes may be of high strategic value in the inhibition of migration of proinflammatory leucocytes into the CNS. The mechanism responsible for the induction of annexin-1 in glial cells during the clinical phase of EAE is not clear. Dexamethasone up-regulates annexin-1 expression in many cell types [10–15], thus annexin-1 immunoreactivity in astrocytes could have been induced by circulating glucocorticoids which reach peak levels during the clinical phase of EAE [4,14], although contradictory results have been published [48].

Promising new therapies for MS are currently being evaluated [49], but glucocorticoids are still used as therapy despite significant side-effects. Investigation of second messenger molecules of glucocorticoids such as annexin-1 may provide valuable information on the mechanism by which glucocorticoids modulate inflammation during MS.

ACKNOWLEDGMENTS

The authors wish to thank Karin Sontrop, Wim van den Bovenkamp and Maarten van der Cammen for their technical assistance and Terence Smith (London, UK) for valuable discussion. This study was initiated by Frank Berkenbosch (who sadly died April 1993). Financial support for this study was provided by the Dutch foundation 'Friends MS Research' (grants 90-67 and 95-204 MS).

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