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Effect of human interferon-alpha-2b on experimental endometriosis in rats: comparison between short and long series of treatment

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ABSTRACT

Objective: A randomised and controlled experimental study was carried out to determine the effect of short and long series of treatment with recombinant human interferon-alpha-2b on surgically induced endometriosis in rats.

Study design: Ninety-six Wistar adult female rats, which had undergone an autotransplant into the peritoneal cavity of four endometrial fragments measuring 4.5 mm at the side, were randomly divided into three groups. One third of the animals were manipulated like the treated animals but were not given treatment and served as control (group C). Another third (group S) were treated with three doses (one every 48 h, 100,000 U per dose) of recombinant human interferon-alpha-2b (subcutaneous route), and the last third (group L) were treated with fifteen doses of interferon (100,000 U every 48 h).

Results: Before interferon was administered, there were no differences between groups in the average growth of experimental endometriosis per animal (17.3 ± 6.7, 18.1 ± 9.2, 16.4 ± 5.6 mm in groups C, S and L respectively). After the treatment, experimental endometriosis per animal was significantly smaller in the groups treated with interferon than in the control non-treated group ($p < 0.001$), and in the group treated with 15 doses versus the group treated with 3 doses ($p < 0.05$), (17.6 ± 7.5, 14.0 ± 9.5, 9.4 ± 6.0 mm in groups C, S, and L respectively). While the implants of the animals in the control group showed no change in size throughout the study (120 days) (+1.96% of variation), the mean size of the implants in the treated rats decreased, (22.7% with the short and 42.8% with the long series of treatment with interferon). Only one implant in group C (0.8%) disappeared, while this occurred in 27 cases (22.5%) in group S ($p < 0.001$) and in 45 (37.5%) in group L ($p < 0.001$ versus group C and $p < 0.05$ versus group S).

Conclusion: The long series of treatment with human interferon-alpha-2b was more effective than the short one in reducing the size of surgically induced endometriosis in the peritoneal cavity of the rat.

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1. Introduction

The growth of endometrial epithelial cells, as well as their differentiation, is directly or indirectly regulated by the stromal cells surrounding them. Furthermore, inflammatory cells, which are often present in the endometrial stroma, can provide additional stimuli for each type of endometrial cell. The fibroblasts of the endometrial stroma differ from those of most tissues, since they have hormonal receptors, and so they show changes according to the endocrinological environment. The hormonal signals are integrated with the messages between cells, which are regulated by cytokines, growth factors and integrins, such that autocrine or paracrine signals contribute to the final amount of cellular growth and differentiation [1].

It is clear that steroidal hormones, growth factors, cytokines, and perhaps another unknown substances, aid the development of ectopic endometrium, but they seem not to be the cause of the endometriosis. Endometriosis is probably due to some deficiencies in the immunological system, or to a higher resistance of the displaced endometrial cells, so that some women may become immunotolerant to elements against which they should be immunocompetent [2].

At this moment, the most urgent question to solve the enigma of endometriosis seems to be the establishment of the order in which all the known immune alterations occur. Moreover, perhaps if we know whether the immune disorders that have been recently

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described for endometriosis are actually implied in its origin, genetic or exogenous, the task of developing a curative treatment for this disease will become easier.

Twenty years ago, Gleicher recommended a change in the direction of therapeutic investigations about endometriosis towards prospective studies with immunological treatments [3]. It is currently held that endometriosis can be vulnerable to immunological manipulation, but there are not clear guidelines in this respect.

The absence of spontaneous endometriosis in animals, except for the rare cases described in some superior primates [4], implies the necessity of performing the research on women. This involves important ethical and methodological drawbacks, and hinders the introduction and trials of new therapies. These difficulties can be avoided by experimental studies using endometrial transplants in laboratory animals. In this sense, the rat model proposed by Vernon and Wilson [5] mimics spontaneous human endometriosis in many respects, and constitutes a good model for research on the effect of the experimental therapies, as we have previously confirmed [6–8].

Some of the biological effects described with the use of interferon alpha (antiviral, antitumor and immunomodulating) are highly suggestive in the search for new approaches in endometriosis treatment. In fact, immunomodulation with interferon could cause largely opposing effects on the different components of the immune system such as those generally observed in patients with endometriosis. When given to a patient after she has been exposed to an antigen, they suppress or reduce the production of antibodies [9], and interferon is also known to modulate positively the cytotoxic capacity of T-cells [10] and the efficacy of NK cells [11], as well as the activity of peritoneal macrophages [12]. Moreover, interferon displays anti-proliferative and cytostatic effects that have been largely demonstrated on a great number of neoplastic processes.

In a previous study, we found that a short series of treatment with recombinant human interferon-alpha-2b reduces the size of surgically induced endometriosis in the peritoneal cavity of Wistar rats [13]. Now, a longitudinal, analytical, controlled, double blind and randomised experimental study was carried out to determine whether a longer series of treatment improves the results versus the short series.

2. Materials and methods

Ninety six virgin, mature, female Wistar rats, weighing more than 190 g and who were about three months old were employed. The animals were maintained on a 14/10 h (light/dark) photoperiod and fed with rat chow and water ad libitum. The vivarium temperature was maintained at 18–25 °C, the relative humidity at 50–60%, and the national standards and guidelines for the care and use of animals were followed rigorously.

Experimental endometriosis was induced following the surgical method described by Jones [14]. Anaesthesia was performed through a droperidol/phentanyl (1/50 Thalamonal®) intramuscular injection, with a dosage of 0.15 mg/kg of phentanyl.

The endometrium obtained after the resection of the left uterine horn, was cut into four fragments measuring 4.5 mm at the side. These fragments were implanted with a suture of polypropylene 5/0, in the cranial and caudal parts of the peritoneal wall to the right and left of the midline laparotomic incision, with a separation of 1.5 cm. This laparotomy, performed to induce experimental endometriosis, was designated as L1.

Between 30 and 45 days after the induction procedure, all the animals underwent a second laparotomy (L2) to determine the growth of each implant. This growth was classified by the measurement of the maximum diameter in mm, as well as by classification in grades of growth based on a modification of the classification proposed by Jones [14] (Table 1). In addition, the growth of the experimental endometriosis in each animal was measured by the sum of the sizes of its four implants (in mm and in grades).

Table 1
Classification of the growth of experimental endometriosis by implant and by animal (in grades).

Grade	Description
Growth of each implant	
0	The implant had disappeared or, if visible, it never became a cyst.
1	It formed a vesicle whose major diameter was <2 mm or if equal to 2 mm it was solid.
2	The implant formed a cyst with fluid and its major diameter was ≥2 mm, but <4.5 mm (not bigger than the initial size).
3	The diameter of the vesicle was similar to, or larger than, the initial size of the implant (≥4.5 mm).
Growth of experimental endometriosis by animal (in grades)	
0–12	A value obtained by the sum of the grades of growth of the four implants of each animal.

At this time the animals were randomly divided into three groups: Group C (Control) ($n = 32$), group S (short series of treatment) ($n = 32$), and group L (long series) ($n = 32$). As group C served as a control group, these rats were not given any treatment but they were manipulated as much as those treated, so that the level of stress due to manipulation was equal in both groups. Moreover, other previous studies reported that the size of the experimental endometriosis did not change by the stress resulting from injecting a placebo substance. The rats in group S were treated with three doses of interferon-alpha-2b (Intron A®, Shering-Plough, Madrid, Spain) (100,000 U every 48 h) by subcutaneous injection of 0.2 ml. In this group the first dose was administered 48 h after L2. The animals in group L were treated with 15 doses using the same method, and for 30 days. Finally, at day 120 from L2, all the rats underwent the last laparotomy (L3).

At the time of any implant evaluation, the researcher did not know the previous growth of the implants or which group the rat belonged to.

In the statistical analysis of the results, analysis of variance (ANOVA) was used, applying Newman–Keuls' test to accomplish the hypothesis contrast. The differences were considered statistically significant when $p < 0.05$.

3. Results

Five animals died before completing the investigation protocol to which they were allocated, and so they were excluded from the study. Mortality seemed related to post-operative depression, since autopsy did not show another cause, and there were no differences between groups. That meant a 5.21% mortality rate, and 1.74% per laparotomy. Thus, the groups ended as follows: group C ($n = 31$), group S ($n = 30$), and group L ($n = 30$).

The average weight of the animals at the beginning of the first laparotomy was 237 ± 29 g. The initial weight and the increase in weight were uniform, and there were no significant differences among groups. Nor were there any significant differences in the mean quantity of anaesthetics given in each laparotomy, or in the time spent for each one (38.1 ± 5.1 min to induce experimental endometriosis (L1), 20.4 ± 3.1 min for L2 and 17.0 ± 3.1 for L3).

Before treatment, at the time of L2, there were no significant differences between groups when we considered the mean size of the implants or the endometriosis growth by animal, in mm and in grades (Table 2), and thus the groups were homogeneous before treatment. When L3 (the last laparotomy) was performed, however, the mean size of the implants, in mm and in grades

Table 2
Mean size of experimental endometriosis by animal, in millimetres and in grades, at the laparotomy before treatment (L2) and at the after treatment laparotomy (L3).

	Group C ($n=31$) $x \pm SD$	Group S ($n=30$) $x \pm SD$	Group L ($n=30$) $x \pm SD$
In millimetres			
L2	17.3 ± 6.7	18.1 ± 9.2	16.4 ± 5.6
L3	17.6 ± 7.5	$14.0 \pm 9.5^{*,+}$	$9.4 \pm 6.0^{*,\&,\#}$
In grades			
L2	8.7 ± 2.0	8.7 ± 2.8	9.2 ± 2.3
L3	8.9 ± 2.5	$6.7 \pm 3.9^{*,+}$	$5.3 \pm 3.3^{*,\&}$

x: Mean size, SD: standard deviation. L2: laparotomy of evaluation of the growth of the implants after induction; L3: final laparotomy (day 120), after treatment.
* $p < 0.001$ between L2 and L3.
+ $p < 0.05$ versus the control group.
& $p < 0.001$ versus the control group.
# $p < 0.05$ between groups L and S.

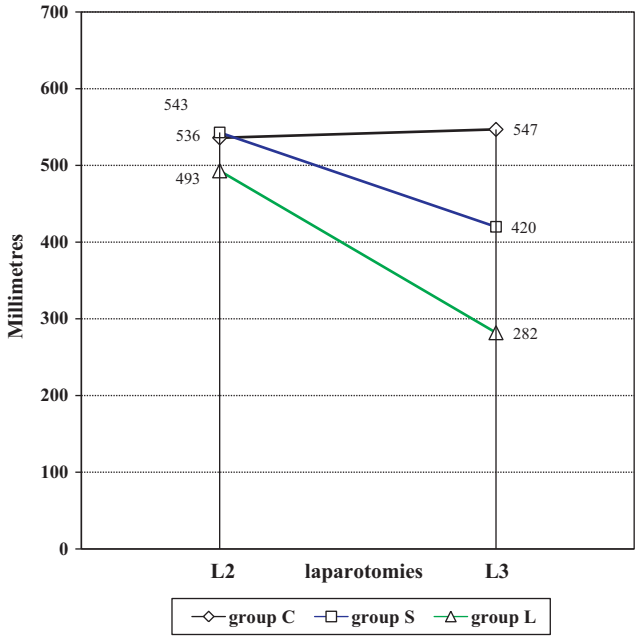

Fig. 1. Change, from L2 to L3, of the value obtained by adding the sizes of all the implants in each group.

by animal, was smaller in the treated groups than in the control group, ($p < 0.05$ group S versus group C, and $p < 0.001$ group L versus C). Furthermore, in the group treated with the long series (group L), the mean size of the implants in mm was smaller than in group S (treated with the short series, $p < 0.05$) (Table 2).

Fig. 1 shows the change, from L2 to L3, of the values obtained by adding the sizes of all the implants in each group. At the time of L3, this value showed no change in the control group (group C, +1.96% of increase). By contrast, this value decreased in the treated groups, this reduction being greater in group L (22.7% reduction in group S, and 42.8% in group L).

Finally, only one implant seemed macroscopically to have disappeared in group C (0.8%) versus 27 (22.5%) in group S ($p < 0.001$) and 45 (37.5%) in group L ($p < 0.001$ for group C and $p < 0.05$ for group S).

4. Comment

The many alterations of the elements of the immunological system which have been found in patients with endometriosis have been integrated from three different points of view. Some authors deduce, from the alterations of the humoral component of the immune system, that endometriosis may be considered as an auto-immune disease [15,16]. This seems to be supported by the presence of organ-specific auto-antibodies (anti-endometrial antibodies) [17,18] and, to a minor degree, of antibodies against cellular components (anticardiolipin antibodies) [19]. The absence of association with some antigens of the HLA system, however, which is a common characteristic of the auto-immune diseases, could make us think that an autoimmune aetiology is less probable for endometriosis [20].

In the second place, there are authors who advocate that an immunological cellular deficit is the basic etiopathological condition that underlies this disease. According to this hypothesis, the disease develops when the cellular immune response fails to eliminate the displaced endometrial fragments. The efforts of the cellular immune system to limit the ectopic endometrial growth, though insufficient for that purpose, play a critical role in the activation of the B cells, which are at first at rest and then reach an activated state with further differentiation and production of antibodies [2].

Finally, the most primitive and non-specific elements of the immune system have also been associated with the aetiology of the disease. According to the theory of macrophage activation as a cause of endometriosis [21], the peritoneal macrophages of women with endometriosis are larger, more mature and hyper-activated. They produce more growth factors and fibronectin. Thus, in the presence of retrograde menstrual flow, an increase in macrophage activation and a relatively hyper-estrogenic state are optimum conditions for the development of endometriosis.

In any event, many of the alterations described in human endometriosis have been demonstrated in experimental models, some of them murine [22,23], which supports the possibility of using these models for immunologic investigations.

Considering the coexistence of the immune alterations described in this disease, there are therefore two main possibilities for immunomodulation: the immuno-stimulative therapy and the immuno-suppressive one. Immuno-suppressive therapy has been proposed by investigators who are dedicated to reproductive medicine, because they are concerned about the consequences that the factors freed in the immune reactions could have on the fertility of these patients. They have experimentally tried corticoids [24], pentoxifylline and verapamil [25], indomethacin [26] and, as a rule, the results reported with these therapies, in terms of recovering the lost fertility, have been good or very good. D'Hoodge et al., however, reported the immuno-suppressive effect of methylprednisolone and azatioprin in baboons with spontaneous endometriosis, and showed that the immunosuppressed animals had a significantly greater number and size of endometriotic implants than the animals in the control non-treated-group [27].

Immuno-stimulative therapy has been less studied for endometriosis, perhaps because it does not seem so interesting for reproductive purposes, although it has been considered of interest to control the clinical symptoms and to avoid progression and recurrence of the disease. Consequently, the possibilities are more limited and involve great complexity. The immune system acts in a complex chain, so that the modulation of one element could alter or affect the entire system. This fact, which would seem to be a major obstacle, could also be advantageous, in the same way as deviations of several of the components of the immune system have been observed in endometriosis, probably having a single origin. Cytokine and chemokine expression in rat endometriosis is similar to that in human endometriosis. Therefore, this model may be useful in the investigation of the pathogenesis and treatment of endometriosis [28]. The modulation of the key element could

establish changes in the network which restore the lost physiological balance of the system. Substances, such as interleukine-2 and interferon, could be tested for this aim.

Previous studies have demonstrated that the human interferon-alpha is substantially active in rats [13,29–31]. Badawy et al. examined the effect of interferon alpha-2b at various concentrations on the growth of endometrioma cells lines in vitro. They demonstrated that interferon alpha-2b inhibits the growth and DNA synthesis of endometrima cells in culture. This effect increased with increasing concentrations of interferon alpha-2b [32]. These findings represent the mainstay to explain the results of our study.

In our work, the short series of treatment with interferon-alpha produced a significant decrease in the size of the implants with respect to the non-treated animals, resulting in an overall reduction of 22.7%, but we found that the long series produced a significantly greater benefit than the short one, leading to an overall reduction of 42.8%. Another finding was the high rate of macroscopic disappearance of implants which occurred in the groups treated with interferon, and this rate was also higher in the long series. Remarkably, this macroscopic disappearance of a significant proportion of implants was sustained in the long term. This fact was of interest in our experimental model because, although the hormonal treatments reached higher rates of macroscopic disappearance, they always recurred when the treatment was discontinued. Our findings therefore suggest the possibility of their definitive disappearance and we have begun new studies in order to confirm this.

In fact, these better results for the longer series of treatment could be foreseen. Spiegel had already indicated some recommendations about the optimal therapeutic management of interferon, and pointed out that repeated doses seemed to be more effective than isolated ones [33]. Keeping in mind that recommendation, we aimed to administer the largest possible number of doses, which we referred to as a long series of treatment, but hypothetically there could be an imposed limitation. Human interferon is a biological product which is antigenically foreign for the rat. Perhaps sensitisation may be possible and, in this case, interferon could become an ineffective treatment because of hypothetical blocking antigen-antibody reactions. Thus, interferon could theoretically be neutralised by specific antibodies resulting in a loss of effectiveness, though practical experience does not seem to confirm this postulate in all the cases. Because of these findings, and as anti-interferon antibodies have been observed in the rat after three intravenous infusions [34], we think that more than fifteen doses would not provide any additional benefit.

With the necessary caution with respect to experimental findings, and keeping in mind that any model has limitations, we think that the results of this initial study confirm the effectiveness of recombinant human interferon-alpha-2b in producing a reducing effect on surgically induced experimental endometriosis in this murine model. In addition we have found that a long series of treatment is more successful than a short series.

In accordance with Gleicher's recommendations, this study will follow a continuous path for further research on immunological therapies for endometriosis.

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