The germinal vesicle of the mouse oocyte contains elements of the phosphoinositide cycle: what is their role at meiosis resumption?

Nathalie Avazeri, Arlette Pesty, Brigitte Lefèvre

To cite this version:
Nathalie Avazeri, Arlette Pesty, Brigitte Lefèvre. The germinal vesicle of the mouse oocyte contains elements of the phosphoinositide cycle: what is their role at meiosis resumption?. Reproduction Nutrition Development, 1998, 38 (6), pp.671-682. hal-00900241

HAL Id: hal-00900241
https://hal.science/hal-00900241
Submitted on 1 Jan 1998

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
The germinal vesicle of the mouse oocyte contains elements of the phosphoinositide cycle: what is their role at meiosis resumption?

Nathalie Avazeri, Arlette Pesty, Brigitte Lefèvre*

Institut national de la santé et de la recherche médicale Unité 355
and Institut fédératif de recherche sur les cytokines, 32, rue des Carnets, 92140 Clamart, France

(Received 1 October 1998; accepted 13 October 1998)

Abstract — The role of the nuclear phosphoinositide (PI) cycle during meiotic resumption in mouse oocytes was examined. First, using indirect immunofluorescence staining with specific monoclonal antibodies (mAbs) against elements of this cycle, the presence of inositol trisphosphate receptors (IP3Rs) (IP3R-1 or IP3R-3) or phosphoinositide-phospholipase (PLC) isoforms (PLCβ1 or PLCγ1) was monitored in the germinal vesicle (GV). Using confocal laser scanning microscopy, we analysed the effects of the nuclear microinjection of these antibodies on both spontaneous nuclear calcium oscillations and meiosis resumption. Immunostainings showed that IP3R-1 and PLCβ1 isoforms were both present in the GV, whereas IP3R-3 and PLCγ1 isoforms were not. The anti-IP3R-1 mAbs or the anti-PLCβ1 mAbs microinjected into the GV induced inhibition of both the nuclear Ca2+ oscillations and the meiotic process, whereas the anti-IP3R-3 mAbs and the anti-PLCγ1 mAbs did not. We concluded that a specific nuclear PI cycle is present in the mouse oocyte and meiosis resumption requires a specific nuclear phosphoinositide-dependent Ca2+ signal. © Inra/Elsevier, Paris

oocyte / nucleus / calcium / IP3 receptors / PLC

Résumé — La vésicule germinative de l’ovocyte de souris contient des éléments du cycle des phospho-inositides : quel est leur rôle dans la reprise de méiose ? Le rôle du cycle des phospho-inositides nucléaires (PI) dans la reprise de méiose a été étudié dans l’ovocyte de souris. Dans un premier temps, la présence et la localisation de certains éléments de ce cycle tels que deux types de récepteurs à IP3 (IP3R-1 et IP3R-3), et deux isoformes de la phospholipase C (PLCβ1 et PLCγ1) ont été recherchés dans la vésicule germinative (GV) par immunofluorescence indirecte grâce à des anticorps monoclonaux spécifiques de chacun d’eux. De plus, nous avons analysé, en microscopie confocale, les effets de la micro-injection nucléaire de ces différents anticorps spécifiques sur les oscillations calciques nucléaires spontanées et la reprise de méiose. L’immunohistochimie a montré que seuls les récepteurs à IP3 de type 1 et la PLCβ1 étaient présents dans la VG tandis que les récepteurs de type 3 et la PLCγ1 ne l’étaient pas. Microinjectés dans la VG, l’anticorps contre le récepteur à IP3 de

* Correspondence and reprints
E-mail: brigitte.lefevre@inserm.ipsc.u-psud.fr
type 1 comme l’anticorps contre la PLCβ1 induisaient une inhibition des oscillations du Ca²⁺ nucléaire et du processus méiotique tandis que les anticorps contre les récepteurs à IP₃ de type 3 ou contre la PLCγ1 n’avaient aucun effet. En conclusion, un cycle des PI nucléaires est fonctionnel dans l’ovocyte de souris et la reprise de méiose nécessite un signal Ca²⁺ nucléaire dépendant des phosphoinositides. © Inra/Elsevier, Paris

ovocyte / noyau / calcium / récepteurs à IP₃ / PLC

1. INTRODUCTION

The precise biochemical pathways that are responsible for inducing the phosphorylation/dephosphorylation cascade that results in meiosis resumption in mammals are still unknown. Many elements have been discovered, at least in rodents, revealing the clear implication of cyclic adenosine monophosphate (cAMP) as a ‘negative’ second messenger whose presence or disappearance in the oocyte plays a major role in meiosis arrest or resumption (for a review, see [10]). Despite the knowledge of these elements, nothing is clearly known concerning a factor of granulosa or cumulus cell origin that can override the inhibitory effects and promote meiotic resumption. For several years now, researchers have been focusing on the role of an intracellular calcium signal as the possible inducer of meiosis resumption. The debate, however, is still ongoing between the defenders of the calcium implication and those who claim that calcium is not essential at this time of meiosis (for review, see [10]). The major argument of the latter group is that buffering the intracytoplasmic calcium by BAPTA (1, 2-bis (o-aminophenoxy) ethane-N, N, N’, N’-tetraacetic acid) has no implication on meiosis resumption in the mouse [3, 29]. We also demonstrated a clear correlation between the acquisition of the meiotic competence during oocyte growth and the acquisition of the full functionality of the IP₃-dependent calcium release [14]. Moreover, we observed that the changes in the distribution of the chromatin in the germinal vesicle (GV) occurring before germinal vesicle breakdown (GVB) were related to the occurrence of these spontaneous oscillations [13], i.e. the oocytes that will resume meiosis were those that exhibited calcium oscillations. Furthermore, having observed that calcium was also oscillating in the nucleus during the first step of meiosis resumption [13], we continued our investigations to determine whether the nuclear Ca²⁺ could play a major role during the GVB. We were impelled in that direction by numerous recent studies on the nucleus of different cell types that demonstrated the presence of elements of the biochemical pathways playing a role in calcium homeostasis (figure 1) such as phosphoinositide-phospholipase C (PLC), inositol trisphosphate receptors (IP₃Rs), and protein kinase C (for reviews, see [16, 24]). Thus, we worked first on localising several isoforms of PLC or IP₃ receptors in the immature mouse oocyte and then on examining their respective importance in the propagation of nucleic Ca²⁺ nuclear and in the meiotic process.

Indeed, spontaneous calcium oscillations occurred in most of the fully grown oocytes in the minutes following their release from the follicles [3, 13, 29]. We also demonstrated a clear correlation between the acquisition of the meiotic competence during oocyte growth and the acquisition of the full functionality of the IP₃-dependent calcium release [14]. Moreover, we observed that the changes in the distribution of the chromatin in the germinal vesicle (GV) occurring before germinal vesicle breakdown (GVB) were related to the occurrence of these spontaneous oscillations [13], i.e. the oocytes that will resume meiosis were those that exhibited calcium oscillations. Furthermore, having observed that calcium was also oscillating in the nucleus during the first step of meiosis resumption [13], we continued our investigations to determine whether the nuclear Ca²⁺ could play a major role during the GVB. We were impelled in that direction by numerous recent studies on the nucleus of different cell types that demonstrated the presence of elements of the biochemical pathways playing a role in calcium homeostasis (figure 1) such as phosphoinositide-phospholipase C (PLC), inositol trisphosphate receptors (IP₃Rs), and protein kinase C (for reviews, see [16, 24]). Thus, we worked first on localising several isoforms of PLC or IP₃ receptors in the immature mouse oocyte and then on examining their respective importance in the propagation of nucleic Ca²⁺ nuclear and in the meiotic process.
2. MATERIALS AND METHODS

2.1. Media

M2 or M16 medium [7] supplemented, respectively, with 4 or 15 mg·mL⁻¹ BSA (fraction V; Sigma, Saint-Quentin Fallavier, France) were used for the oocytes culture. The microinjection medium was 140·10⁻³ mol·L⁻¹ KCl, 10⁻³ mol·L⁻¹ MgCl₂, 5·10⁻³ mol·L⁻¹ HEPES, pH 7.2.

2.2. Oocyte recovery

Oocyte-cumulus cell complexes were isolated from the antral ovarian follicles of 5-week-old C57 females (Charles River, Saint-Aubin-les-Elbœuf, France). Ovarian stimulation was induced by injection of 5 IU of a pregnant mare's gonadotropin serum (Chronogest, Intervet International, Boxmeer, Holland). The ovaries were removed 48 h later, placed in a Petri dish containing M2 medium and the antral follicles were disrupted with sterile needles. Then cumulus-enclosed oocytes were collected and freed from surrounding cells by repeated aspiration with a glass capillary in the M2 medium.

2.3. Immunolocalisation of IP₃-receptors and PLC isoforms

For localisation in whole oocytes, the zona pellucidae were removed in a solution of α-chymotrypsin (0.1 %) in PBS supplemented with BSA (3 %). The oocytes were then treated as already described [21]. Finally, the immunostained oocytes were observed by confocal microscopy using a 40 × objective (NPL Fluotar 40/0.70) at a single optical plane through the GV. Images were produced by an average of six consecutive frames using the on-line Kalman algorithm.

For immunolocalisation in isolated nuclei, suction was applied with a very thin micropipette on the cytoplasmic membrane of the oocytes, to obtain its mechanical disrupter. Then, the isolated nuclei were treated as already described and observed with a 60 × objective (PlanApo, 60/0.95) [21].
2.4. Experimental protocols

The experimental protocols are summarised in figure 2. As soon as the oocytes were recovered, either the agonist or the antagonists of the phosphoinositide turnover, diluted in the microinjection medium, were microinjected into the GV. The microinjections were performed either after Ca^{2+} indicator loading to record the immediate calcium events or without loading to study the GVB kinetics for 4 h in culture. The agonist used was the inositol 1,4,5-trisphosphate (IP_3, 10^{-6} mol-L^{-1}; Calbiochem, San Diego, CA, USA); the antagonists were the low molecular weight heparin (2 \times 10^{-6} mol-L^{-1}), which binds non-specifically to the IP_3Rs (Calbiochem), two monoclonal antibodies (mAbs) against the IP_3Rs (the anti-IP_3 type 1 isoform, 1:20, anti-IP_3 R-1 mAbs; Calbiochem, and the anti-IP_3 type 3 isoform, 1:20, anti-IP_3 R-3 mAbs; Transduction Laboratories, Lexington, KY, USA) and two mAbs against PLC isoforms (the anti-PLCβ1, 1:20, and the anti-PLCγ1, 1:20; UBI, Santa Cruz, CA, USA). Table I gives the specificity of all the antibodies used.

2.5. Fluorescent Ca^{2+} indicator loading of the oocytes

The cell-impermeant Oregon Green 488 BAPTA-1 dextran (Oregon green dextran; Molecular Probes Inc., Eugene, OR, USA) was used to load the nucleus since its high molecular weight (70 000 MW) circumvents it from passing through any membrane. It was diluted temporarily in the microinjection medium, then injected into the GV of the oocytes.

2.6. Confocal microscopy of living oocytes

All measurements of fluorescence emission were performed using a Bio-Rad MRC600 confocal laser scanning station interfaced with a Nikon Diaphot inverted microscope as described previously [14]. In brief, to perform time-lapse calcium imaging studies, dye-loaded oocytes were viewed through a 20× or a 40× objective (Fluor 20/0.75; NPL Fluotar 40/0.70) at a single optical plane through the GV. Calcium kinetic images were resolved by monitoring the oocytes with a 2-s interval between two consecutive acquisitions. Sequential digitized images were recorded for later analysis. Fluorescence intensity was measured in a rectangular area positioned in the nucleoplasm excluding the nucleolus. On the kinetic curves, data are expressed in arbitrary units uncalibrated in terms of calcium concentration.

3. RESULTS

3.1. Immunolocalisation of IP_3 receptors and PLC isoforms

3.1.1. Whole oocytes

Immunostaining with anti-IP_3 R-1 mAbs revealed not only fluorescence located on

![Figure 2](image-url)
the cytoplasmic network but also a high fluorescence intensity associated with the nuclear membrane (figure 3A). Using anti-IP$_3$R-3 mAbs, immunohistochemistry also evoked a localisation of IP$_3$R-3 on the cytoplasmic network, with higher fluorescence in the vicinity of the plasma membrane, but the nucleus area was not stained (figure 3B). The examination under confocal microscopy of oocytes immunostained with the anti-PLC$_{β1}$ mAbs showed an important nuclear staining. The cytoplasm was slightly fluorescent whereas a positive signal was also detected at the plasma membrane level (figure 4A). When the anti-PLC$_{γ1}$ antibody was applied, the presence of this PLC isoform was always revealed both in the cytoplasm and in the nucleus, with a higher fluorescence intensity in the cytoplasm (figure 4B).

3.1.2. Isolated nuclei

The observations of the immunostained isolated nuclei confirmed the previous data obtained in the whole oocytes. IP$_3$ receptors of type 1 were localised at the nuclear membrane level (figure 3C). No IP$_3$R-3 were found in the nucleus, and even if a slight signal appeared in the vicinity of the nuclear membrane, it was not significant compared to the signal obtained with IP$_3$R-1 (figure 3D). The PLC$_{β1}$ isoform was present mainly on the nuclear membrane and around the nucleolus and slightly in the nucleoplasm (figure 4C). Even though the PLC$_{γ1}$ isoform was slightly detectable on the nuclear membrane and around the nucleolus, the fluorescence intensity was very weak and almost insignificant compared to that obtained with the other PLC isoform (figure 4D).

3.2. Effects of the PI turnover agonist or antagonists on spontaneous calcium oscillations

The microinjection of the calcium indicator, oregon green dextran, into the GV induced first, an important release of calcium and then, the spontaneous nuclear Ca$^{2+}$ oscillations progressively became firmly established (figure 5A). In figure 5B, the images show a nuclear calcium wave, the dextran-conjugated probe excluding the nucleolus.

Low molecular weight heparin induced a very fast decrease in nuclear Ca$^{2+}$ oscillations into typical small fluctuations of basal fluorescence. Only some spikes of reduced amplitude appeared with irregular frequency for the following 30 min (figure 6A).

A low percentage of oocytes does not exhibit calcium oscillations after their release from the follicle. The microinjection of exogenous IP$_3$ into the GV of these non-oscillating oocytes induced first irregular, then regular, nuclear Ca$^{2+}$ oscillations (figure 6B).

<table>
<thead>
<tr>
<th>mAbs</th>
<th>Laboratory</th>
<th>Specificity</th>
<th>Cross-reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-IP$_3$R-1</td>
<td>Calbiochem</td>
<td>C terminus domain</td>
<td>Human, rabbit, pig, mouse and bovine</td>
</tr>
<tr>
<td>Anti-IP$_3$R-3</td>
<td>Transduction</td>
<td>N terminal domain</td>
<td>Bovine, rat, mouse and human</td>
</tr>
<tr>
<td>Anti-PLC$_{β1}$</td>
<td>UBI</td>
<td>PLC$<em>{β1}$ (150 kDa) also 100 kDa and 41 kDa fragments of PLC$</em>{β1}$</td>
<td>Bovine, rat, mouse and human</td>
</tr>
<tr>
<td>Anti-PLC$_{γ1}$</td>
<td>UBI</td>
<td>PLC$<em>{γ1}$ and with some non-PLC$</em>{γ1}$ SH-3 containing proteins.</td>
<td>Rabbit, rat, mouse and human</td>
</tr>
</tbody>
</table>
The microinjection of anti-IP$_3$R-1 mAbs into the GV induced an important release of nuclear calcium which was not uptaken again. The fluorescence level remained high and nuclear Ca$^{2+}$ oscillations did not reappear in half of the studied oocytes, whereas the other half exhibited only a transitory disorganisation of the signal (figure 7A). The microinjection of anti-IP$_3$R-3 mAbs, however, was without effect on nuclear calcium oscillations (figure 7B).

In most of the cases, the microinjection of the anti-PLC$\beta$1 mAbs seemed to inhibit the nuclear Ca$^{2+}$ oscillations, whereas the nuclear microinjection of anti-PLC$\gamma$1 mAbs did not affect the spontaneous nuclear calcium events.

### 3.3. Implication of the nuclear phosphoinositide cycle in meiosis resumption

When the anti-IP$_3$R-1 mAbs was microinjected into the GV, the meiotic process was strongly inhibited: the GVB rate remained low and constant during the 4 h in culture, compared to the oocytes in the control group ($P < 0.05$) (figure 8A).
Oocytes receiving a microinjection of anti-IP₃-R-3 mAbs into the nucleus resumed meiosis, as did the control group oocytes (figure 8B).

When the anti-PLCβ1 mAbs was microinjected into the nucleus, the GVB kinetics slowed down compared to control group oocytes (figure 8C) with a GVB rate at 4 h still significantly lower.

Anti-PLCy1 mAbs microinjected into the GV had no effect on the GVB process (figure 8D).

4. DISCUSSION

Our results demonstrate for the first time that, in the mouse oocyte, a specific nuclear IP₃-dependent Ca²⁺ signal does exist, and that the spontaneous calcium events observed in the GV immediately after follicular release play a role in the meiotic process since its inhibition induces a dramatic decrease in the GVB rate. Indeed, IP₃ directly microinjected into the GV induces nuclear Ca²⁺ oscillations in the oocytes that did not previously exhibit spontaneous Ca²⁺
**Figure 5.** Control nuclear calcium oscillations exhibited by oocytes loaded with oregon green dextran. To perform time-lapse calcium imaging studies, dye-loaded oocytes were viewed through a $40 \times$ objective at a single optical plane through the germinal vesicle (GV) and nucleolus. Calcium kinetic images were resolved by monitoring the oocytes continuously with a 2-s interval between two consecutive acquisitions. Fluorescence intensity was measured in rectangles of similar areas positioned in the cytoplasm and the nucleoplasm excluding the nucleolus. On the kinetic curves, data are expressed in arbitrary units uncalibrated in terms of calcium concentration. (A) The microinjection of the calcium indicator, oregon green dextran, into the GV induced first an important release of calcium and then, the spontaneous nuclear Ca$^{2+}$ oscillations progressively became firmly established. (B) The images showed a single nuclear calcium wave, the dextran-conjugated probe excluding the nucleolus.

**Figure 6.** Microinjection of IP$_3$ or low molecular weight heparin into oocytes loaded with oregon green dextran. The oocytes were maintained in a 30 µL drop of M2 medium under paraffin oil. Oregon green dextran was first microinjected into the nucleus: (A) When the oocytes exhibited regular spontaneous calcium oscillations, heparin, diluted in the microinjection medium ($2 \times 10^{-3}$ mol·L$^{-1}$), was injected into the germinal vesicle (GV). The regular nucleoplasmic calcium oscillations disappeared towards the baseline with some irregular spikes. (B) When the oocytes did not exhibit spontaneous calcium oscillations, IP$_3$, diluted in microinjection medium ($10^{-6}$ mol·L$^{-1}$), was injected into the GV. Spontaneous nuclear calcium signals recorded in a single optical section showed irregular spikes at first and then increasingly regular oscillations similar to those usually observed in fully grown oocytes.
Figure 7. Blockage of the activation of type 1 or type 3 nuclear IP$_3$ receptors on the calcium signal. Oregon green dextran was first microinjected into the nucleus of the oocytes maintained in an M2 medium drop and the fluorescence intensity was recorded. When spontaneous calcium oscillations occurred, either anti-IP$_3$ R-1 or R-3 mAbs diluted in microinjection medium (1:20) was microinjected into the germinal vesicle (GV): (A) The nuclear calcium concentration remained high and spontaneous oscillations did not appear again in most of the oocytes when anti-IP$_3$ R-1 mAbs was injected. (B) The nuclear oscillations remained regular after an anti-IP$_3$ R-3 mAbs nuclear microinjection.

Figure 8. Effects of the nuclear phosphoinositide (PI) turnover antagonists on the germinal vesicle breakdown (GVB) kinetics. The frequencies of oocytes resuming meiosis were calculated on the total number of studied oocytes (n) from at least three experiments (* P < 0.05). Microinjection of medium alone into the germinal vesicle (GV) (n = 54) did not affect the GVB rate as compared to untreated oocytes (n = 92). The GVB process was inhibited when anti-IP$_3$ R-1 (A) was microinjected into the GV (n = 22); it remained almost unaffected by microinjection of anti-IP$_3$ R-3 (B) into the GV (n = 20). The GVB process was inhibited when anti-PLCβ1 (C) was microinjected into the GV (n = 72), whereas it remained almost unaffected when anti-PLCγ1 (D) was microinjected into the GV (n = 72).
oscillations, while the injection of heparin, which is known to bind non-specifically to IP₃ receptors, into the nucleus of spontaneously oscillating oocytes inhibits the Ca²⁺ oscillations.

As for IP₃R isoforms [19, 22, 30, 31], the presence of β1 and γ1 isoforms of PI-PLC has been demonstrated in the nucleus of several cell types [1, 5, 12]. Thus, we verified the presence of these different isoforms in our cellular model. In the mouse oocyte, we found that the nuclear IP₃-dependent Ca²⁺ release occurs through Ca²⁺ channels associated with type 1 IP₃ receptors. Indeed, both nuclear Ca²⁺ oscillations and GVB are inhibited when a monoclonal antibody, which blocks the activation of type 1 receptors, is injected into the nucleus. These observations indicate that IP₃ receptors are present in the GV of the mouse oocyte as already demonstrated in the Xenopus oocyte [2, 15, 26] and that, at least, type 1 is functional and type 3 is either absent or has a lower affinity for IP₃ than type 1. These data also establish that the nuclear IP₃-dependent Ca²⁺ oscillations observed in the preliminary stages of meiosis resumption are necessary for that process. Moreover, nuclear Ca²⁺ oscillations are regulated by at least one PLC isoform present in the nucleus of fully grown immature mice oocytes, the PLCβ1. Indeed, the nuclear microinjection of anti-PLCβ1 mAbs inhibits the GVB process, whereas the nuclear microinjection of anti-PLCγ1 mAbs has no effect.

Furthermore, our immunofluorescent images provide complementary evidence for the existence of a specific nuclear PI turnover. The staining of whole oocytes and isolated nuclei with anti-IP₃R-1 mAbs reveals a perinuclear repartition of type 1 receptors, as for the Xenopus oocyte [20]. Moreover, this study allows us to specify that IP₃R of type 3 are absent from the GV since no staining of the nuclear area is noticeable on the immunofluorescent images of whole oocytes and the staining of the isolated nuclei is almost insignificant. The presence of PLCβ1 is revealed both in the cytoplasm and in the nucleus, with a higher fluorescence intensity in this last cellular compartment as already observed in rat liver nuclei [1]. The tight association of the PLCβ1 isoform with the nuclear envelope revealed by the immunostained isolated nuclei is consistent with the fact that this isoform possesses a long COOH-terminal sequence which is responsible for its association with the nucleus [12, 18]. When whole oocytes are immunostained with the anti-PLCγ1 mAbs, the nuclear area is not stained. On isolated nuclei, the PLCγ1 specific fluorescence is mainly around the nucleus and the nuclear membrane, but no staining is detected in the nucleoplasm. The intensity, however, is very weak compared to that obtained with anti-PLCβ1 antibodies.

To conclude, in the fully grown mouse oocyte, the free nuclear Ca²⁺ level appears to be regulated by a specific nuclear pathway since the nucleus has the key enzyme (PLCβ1) and the IP₃Rs [4, 11, 15, 16, 21] is necessary for Ca²⁺ release. The implication of nuclear calcium in the meiotic process has already been demonstrated in non-mammalian species. In starfish oocytes, an increase in nuclear Ca²⁺ is hormonally regulated and is required for reinitiating meiosis [25]. In the sea urchin, fertilisation provokes a transient increase in free calcium in the egg nucleus [8, 27]. What could be the role of these nuclear calcium oscillations? The fact that calcium mobilisation is required for vesicle fusion during nuclear reconstruction in Xenopus eggs [28] permits us to hypothesise a role in the breakdown of the nuclear envelope. It could also be responsible for regulating gene expression as previously demonstrated in other cells, the specificity being encoded by the oscillation frequency [6]. Moreover, both cytoplasmic and nuclear calcium are probably implicated in the cascade of phosphorylation/dephosphorylation of the cell cycle proteins since the role of calcium during mitosis has begun to be well documented (for review, see [23]).
ACKNOWLEDGEMENTS

This work was supported by the Institut fédératif de recherches sur les cytokines (Université Paris-Sud, France). We are grateful to Deborah Palmer for her assistance with the English translation and her helpful comments on the manuscript.

REFERENCES


