Short paper

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Effect of interferon-gamma and tumor necrosis factor-alpha on lymphoepithelial interactions within thymic nurse cells*

Isolated thymic nurse cells (TNC) represent a specialized microenvironment *in vivo* where thymocytes interact specifically with subcapsular epithelial cells. They are thought to play a critical role in the process of T cell differentiation. We demonstrate that recombinant murine interferon-γ and recombinant human tumor necrosis factor-α can act on these interactions: they stimulate TNC-derived epithelial cells to establish interactions with thymocytes *in vitro* and to form new lymphoepithelial complexes. This phenomenon is partially inhibited by anti-Ia monoclonal antibodies. Implications of these findings for normal intrathymic differentiation are discussed.

1 Introduction

Thymic nurse cells (TNC) have been considered as a specialized *in vivo* microenvironment [1–3], playing putatively a role in the process of T cell differentiation [1, 4]. TNC are abnormal under several pathological conditions, *i.e.* in obese strain of chicken which develop autoimmune thyroiditis [5], and during the pathogenesis of thymic lymphomas in mice [6–8]: in particular, during the period preceeding the emergence of radiation-induced thymic lymphomas in mice, their epithelial component has lost the capacity to interact with normal thymocytes [9]. Grafting BM cells after a split dose lymphomagenic irradiation restores this capacity [9] as well as it inhibits the development of lymphomas [10]. Interestingly, an anti-lymphomatous protection can also be obtained by repeated injections of recombinant murine (rm)IFN-γ and/or recombinant human (rh)TNF-α after irradiation [11]; these cytokines might act through the restoration of epithelial cells, suggesting that they also play a role in the physiological control of intra-TNC differentiation, via the regulation of lympho-epithelial interactions.

In this report, using an *in vitro* assay of TNC formation, we demonstrate that rmIFN-γ and/or rhTNF-α stimulate the TNC-derived epithelial cells to interact with immature thymocytes. Anti-Ia mAb inhibit the TNC formation *in vitro*, whether or not the epithelial cells are stimulated with rmIFN-γ and/or rhTNF-α. However, although rmIFN-γ increases the expression of class II molecules at the surface of cultured TNC-derived epithelial cells, rhTNF-α, on the contrary, does not change the expression of Ia antigens in this culture system.

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Abbreviations: **TNC:** Thymic nurse cell **rMu IFN-γ:** Recombinant murine IFN-γ **GM-CSF:** Granulocyte-monocyte CSF

2 Materials and methods

2.1 Animals, isolation of TNC

One-month-old C57BL/Ka mice were raised in our animal colony. They were a gift from Drs. H. S. Kaplan and M. Lieberman, Stanford University, Stanford, CA. TNC were obtained by enzymatic dissociation of pooled thymuses and repeated sedimentations, as described [1, 7].

2.2 Cultures

Isolated TNC were grown overnight in RPMI 1640 culture medium (Gibco Bioculture Ltd, Gent, Belgium) supplemented with 10% heat-inactivated FCS. The released lymphocytes were discarded. The epithelial cells were cultured for an additional period of 3 to 4 days during which they were stimulated either with rmIFN-γ (200 U/ml), rhTNF-α (2000 U/ml), or with both (generously provided by Boehringer Ingelheim International, Vienna, Austria). As controls, cultured TNC-derived epithelial cells were incubated either in complete medium without any additive or with denaturated cytokines (rmIFN-γ or rhTNF-α heated at 100 °C for 10 min) or with rhIL 2 (100 U/ml and 1000 U/ml; Glaxo S.A., Geneva, Switzerland).

2.3 *In vitro* reconstitution of lymphoepithelial complexes**

The TNC-derived epithelial cells cultured as described above were treated with trypsin (0.25% in PBS) for 15 min at 37 °C and resuspended in RPMI 1640 culture medium. They were mixed with thymocytes obtained from 16- or 17-day-old fetal thymuses in a ratio of one epithelial cell for

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10 thymocytes. Aliquots of 20 µl of this cell suspension were incubated for 6 h in an inverted Terasaki plate at 37 °C in a 5% CO_2 atmosphere. The percentage of epithelial cells forming lymphoepithelial complexes in such experimental conditions was then estimated [9]. mAb to Ia determinants [mouse IgM anti-I-A^b clones B17-263.R1 and B17-123.R2 (Serotec, Wiesbaden, FRG) and rat IgG anti-mouse Ia antigens, clone M5/114 (Hybritech, San Diego, CA)] were added to the hanging drop culture at a concentration of 200 µg/ml.

2.4 Ia expression determination

Class II molecules were recognized by an anti-Ia mAb (mouse IgM anti-I-A^b clone B17-263-R1, Serotec) which was revealed by a fluoresceinated anti-mouse IgM antibody (goat anti-mouse IgM; Nordic, Leuven, Belgium). For staining, trypsinized epithelial cells were suspended in RPMI 1640 containing 5% FCS. Appropriate amounts of anti-Ia were added to 10^6 cells in 50 µl and incubated at 4 °C for 30 min. After washing in FCS, they were treated with FITC-anti-mouse IgM. The cells were washed in FCS and fixed in PBS containing 1% paraformaldehyde. The percentages of Ia^+ cells were measured with a fluorescence-activated cell sorter (FACS 4, Becton Dickinson, Sunnyvale, CA).

2.5 Statistical analysis

The analysis of variance test (ANOVA) was used to compare the differences between the various experimental groups.

3 Results

3.1 Influence of rmIFN-γ and/or rhTNF-α on TNC lymphoepithelial interactions

As shown earlier [9], 25 ± 5% of the normal, non-stimulated TNC-derived epithelial cells formed lymphoepithelial complexes with 16–17-day-old fetal thymocytes [9]. This percentage strikingly increased when the epithelial cells had been cultured for 3–4 days in the presence of cytokines before the reconstitution assay. The values observed were 54 ± 11% after treatment with rmIFN-γ, 46 ± 9% after rhTNF-α and 56 ± 15% after incubation with both cytokines ($p < 0.005$; Fig. 1).

As control, the TNC-derived epithelial cells were cultured either with heat-denaturated cytokines or with rhIL 2: no effect upon their capacity to reform new lymphoepithelial complexes was seen (data not shown).

3.2 Inhibition of *in vitro* lymphoepithelial complexes formation with anti-Ia mAb

Cytokine-treated or control TNC-derived epithelial cells were tested for their capacity to form *in vitro* complexes with immature thymocytes in the presence of anti-Ia mAb. As shown in Fig. 2, these antibodies partially inhibited the capacity of TNC-derived epithelial cells to form *in vitro*

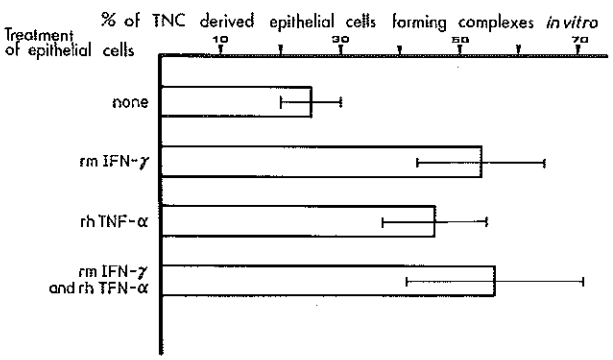

Figure 1. Effect of rmIFN-γ and rhTNF-α on the capacity of TNC-derived epithelial cells to form lymphoepithelial complexes *in vitro*. TNC-derived epithelial cells were cultured for 5 days in the presence of rmIFN-γ (200 U/ml), rhTNF-α (2000 U/ml) or of both cytokines, and tested for their capacity to form lymphoepithelial complexes with 16–17-day-old fetal thymocytes. Results are expressed as percentage of epithelial cells forming new TNC. They represent the mean values and SD obtained from five separate experiments each involving three to five assays.

complexes, whether they were preincubated with cytokines or untreated. The proportion of epithelial cells forming complexes under these conditions was 10 ± 6% for control epithelial cells, 18 ± 6% for rmIFN-γ-stimulated epithelial cells, 6 ± 4% for those incubated with rhTNF-α and 9 ± 3% for those treated with both cytokines. The values observed in the presence of anti-Ia mAb were significantly different from those measured without mAb ($p < 0.005$).

3.3 Expression of Ia antigens on the surface of TNC-derived epithelial cells

When cultured in normal medium for 5 days, 26% of TNC-derived epithelial cells expressed Ia antigens (Fig. 3).

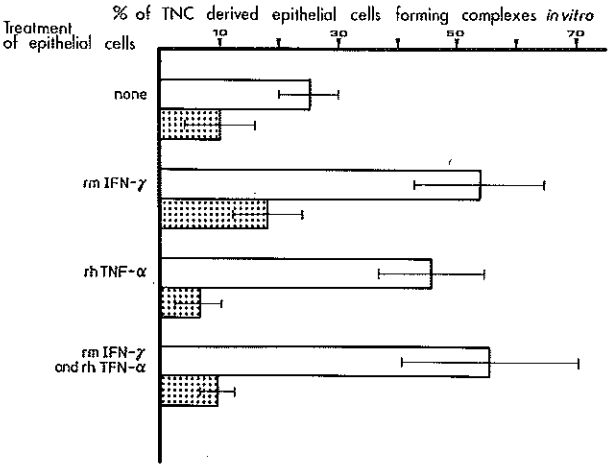

Figure 2. Effect of anti-Ia mAb on the capacity of TNC-derived epithelial cells to form lymphoepithelial complexes *in vitro*. TNC-derived epithelial cells cultured with or without cytokines were tested for their capacity to form lymphoepithelial complexes *in vitro* in the presence (▦) or absence (□) of anti-Ia mAb (200 µg/ml). Results are expressed as percentage of epithelial cells forming new TNC. They represent the mean values and SD obtained from three separate experiments each involving three to five assays.

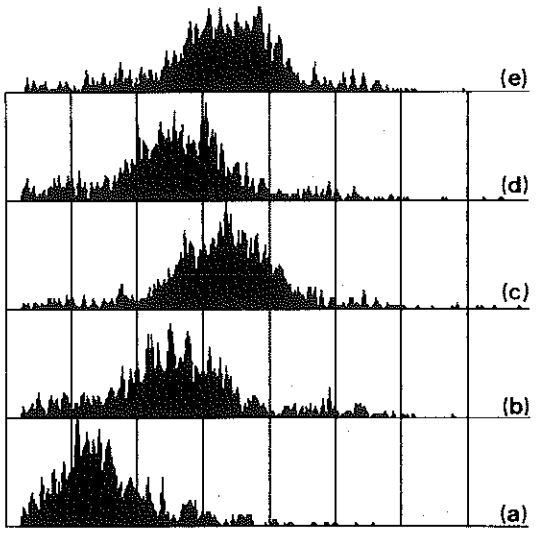

Figure 3. Expression of Ia antigens on the surface of TNC-derived epithelial cells cultured for 5 days. (b) Ia were significantly expressed on 26% of cultured TNC-derived epithelial cells. (c) The expression of Ia antigens increased after stimulation with rmIFN-γ: 62% of TNC-derived epithelial cells are positive. (d) rhTNF-α did not exert any effect on the Ia expression: both profiles of unstimulated TNC and of rhTNF-α were almost identical. (e) After stimulation with rmIFN-γ and rhTNF-α, the profile of Ia expression is similar to that observed after stimulation with rmIFN-γ alone. (a) Negative control.

This percentage significantly increased ($p < 0.005$) when these cells were stimulated either with rmIFN-γ or with rhTNF-α and rmIFN-γ: > 60% of the cells were positive. On the contrary, a stimulation with rhTNF-α alone did not change Ia expression: the FACS profiles were almost similar to those of non-stimulated TNC-derived epithelial cells.

4 Discussion

This study demonstrates that IFN-γ and TNF-α stimulate the capacity of TNC-derived epithelial cells to establish interactions with immature thymocytes. The percentages of TNC-derived epithelial cells forming complexes with immature thymocytes in hanging drop cultures were about 25% under standardized conditions [9]; these percentages were twice as high when TNC-derived epithelial cells had been incubated with rmIFN-γ and/or with rhTNF-α before the co-culture with thymocytes.

The *in vitro* TNC formation and the effects of IFN-γ and of TNF-α upon this phenomenon were partially inhibited by anti-Ia mAb. Ia molecules might thus facilitate cell-cell interactions within TNC, since they are known to act on cell adhesion in other experimental systems [12]. IFN-γ and TNF-α might thus regulate the lympho-epithelial interactions within TNC via the Ia expression. The finding that IFN-γ increased the expression of class II MHC molecules at the surface of cultured TNC-derived epithelial cells supports this hypothesis. This cytokine modulates the Ia expression at the surface of several cell types [13] including thymic accessory cells [14] and thymic epithelial cells [15–17]. However, this mechanism does not explain the mode of action of TNF-α on thymic epithelial cells: indeed, TNF-α stimulated the *in vitro* TNC formation although it did not modify the Ia expression of the epithelial cells, as measured by FCM. A similar discrepancy of the effects of TNF-α and IFN-γ was previously reported in studies on the regulation of lymphocyte adhesiveness to venular endothelium. This process was activated by IFN-γ or TNF-α [18, 19]; however, IFN-γ clearly increased the expression of "high endothelial venules-specific cell-surface antigens" in endothelial cell cultures, but TNF-α did not [20], suggesting that the stimulatory effect of TNF-α was mediated by still unknown molecules. In our system, a similar mechanism might be involved. Moreover, as the effects of TNF-α were inhibited by anti-Ia antibodies, class II molecules might be necessary but not sufficient for binding thymocytes. TNF-α might thus induce the expression of a "binding molecule" whose function depends upon the cooperation with Ia determinants; this molecule has not yet been characterized.

The present findings may be relevant to understand intrathymic lymphopoiesis. First of all, there is some evidence, although still preliminary, that IFN-γ and TNF-α can be produced by thymic cells. IFN-γ was found in the SN of mitogen-activated thymocytes [21], and of thymic stromal cultures [14]. Cells located at the cortico-medullary junction, probably MΦ (Stutman O., personal communication), were shown to produce TNF-α. The release of both cytokines *in situ* might be of importance to maintain the normal functional levels of thymic epithelium [15] and to stimulate the lymphoepithelial interactions responsible for the formation of TNC and, hence, in the production of $CD4^+CD8^-$ thymocytes [22]. Moreover, they could also be responsible for the positive selection of T lymphocytes (for review see [23]), as well as for their negative selection [24].

Class II molecules are not involved in all the thymocyte-epithelial cell interactions. The binding of human thymic epithelial cells to thymocytes in a "rosette formation" assay was inhibited by anti-CD2 and anti-LFA-3 antibodies [25, 26]. However, it was not affected by anti-class II MHC antibodies. However, the epithelial cells used in the mentioned studies, although cortical in origin, did not express A2B5 as did TNC-derived epithelial cells [27]. It cannot be ruled out that different mechanisms are involved in the binding of thymocytes to the various populations of cortical epithelial cells assumed to control different steps of intrathymic lymphopoiesis. The finding that injections of mAb against CD2 do not interfere with postnatal T cell differentiation *in vitro* [28], whereas antibodies against MHC class I and II [29, 30], TcR β chain [31, 32], or CD3 [33] lead to an arrest of intrathymic differentiation at the transition for immature CD4CD8 double-positive to single-positive mature thymocytes, supports this hypothesis. Moreover, the maturation arrest of thymocytes after treatment with antibodies against TcR or against CD3 correlates with a nearly complete abrogation of thymocyte-epithelial cell interactions within TNC [33], indicating that TcR/MHC interactions at this level are necessary for a maturation step. We propose that the local production of cytokines, such as IFN-γ or TNF-α, might interfere with this process.

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