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Antigen-induced inhibition of autoimmune response to rat male accessory glands: distinct characteristics of I-A- and I-E-positive peritoneal cells*

The present report describes different aspects of two populations of peritoneal cells (PC) obtained from rats injected i.p. 2 h or 24 h previously with a suppressor dose of a purified fraction (FI) of rat male accessory glands (RAG) (FI-PC2h and FI-PC24h, respectively). The FI-PC2h, which are mainly I-E (OX17) positive and can suppress the autoimmune response to RAG autoantigens, have an elevated phagocytic activity against *Candida albicans* and capacity to reduce the dye nitroblue tetrazolium. In contrast, FI-PC24h, which are mainly I-A (OX6) positive and can potentiate the autoimmunity to RAG autoantigens, have a diminished capacity to reduce the dye and a diminished phagocytic activity. Moreover, the *Toxoplasma gondii* appear to have a different effect on both populations. The parasites can invade FI-PC2h while FI-PC24h offer resistance to *T. gondii* aggression.

FI-PC2h cultured during 22 h (FI-PC2-24h *in vitro*), or PC obtained from syngeneic recipients injected i.p. 22 h previously with FI-PC2h (FI-PC2-24h *in vivo*) show, as FI-PC2h, an increase of the I-E$^+$ cells and capacity to induce suppression of the delayed-type hypersensitivity response to RAG autoantigens when they are injected to syngeneic rats 10 and 3 days prior to the immunization with chemically modified (diazotized arsanilic and sulfanilic acid) RAG in complete Freund's adjuvant.

The PC obtained 24 h after injection of irradiated rats with N-PC plus FI show an increase of I-E$^+$ cells whereas an enhancement of I-A$^+$ cells can be observed when the PC are obtained 24 h after injection of irradiated and bone marrow-reconstituted rats with N-PC plus FI.

These findings appear to indicate that FI-PC2h and FI-PC24h are functionally different and that the population obtained 24 h after injection of FI of RAG could not originate from either the population present 2 h after injection of FI of RAG injection nor from normal PC. They appear to require bone marrow precursors.

1 Introduction

It is a central axiom of immunology that the antigen-specific receptors of T cells recognize and respond to antigen only when it has been processed and presented by APC [1]. Several reports [2–4] have demonstrated that T_s cells, like T cells responsible for the positive immune response, require antigen processing and presentation by APC to generate H-2-restricted responses. The phenotypic and functional differences between the MΦ which induce T_h *vs.* T_s cell response are not clearly understood. A few reports [5–6] have indicated that the antigen-presenting MΦ populations required for T_h cell induction and those involved in T_s cell induction could be functionally distinguished.

In previous studies we have developed an experimental model of suppression of autoimmune response to chemically modified rat male accessory gland saline extract (MRAG) by i.p. administration of low doses of fraction I (Sephadex G-100) FI of RAG prior to the immunization with MRAG in CFA. The unresponsiveness was specific for the autoantigen at both the cellular and humoral levels [7]. The cell types involved in the suppression included MRAG-specific, cyclophosphamide (CY)-sensitive, inducer-phase T_s cells. Besides CY- and irradiation-sensitive, effector-phase suppressor cells and APC were involved in the suppression [8–10]. Moreover, it was demonstrated that APC obtained 2 h after FI of RAG injection (FI-PC2h), most of them I-E$^+$, were involved in the inducer-phase T_s cell induction, this effect being blocked by anti-I-E pretreatment [11]. In contrast, the APC obtained 24 h after injection of FI of RAG injection (FI-PC24h), most of them I-A$^+$, were involved in the induction of T cells responsible

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Abbreviations: RAG: Rat male accessory glands saline extract **MRAG:** Chemically modified RAG **FI:** Fraction I (Sephadex G-100) of RAG **PC:** Peritoneal cells

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for autoimmune response, this effect being blocked by anti I-A pretreatment. Thus, we could obtain, by i.p. injection of a suppressor dose of FI of RAG, two populations of PC with immunologically opposite characteristics depending on the time of obtention. These functional differences suggest the existence of either unique MΦ subpopulations or distinct activation/differentiation states of these cells.

In an attempt to analyze the functional characteristics of the FI-PC2h and FI-PC24h, we studied in this report their phagocytic capacity towards *Candida albicans* and *Toxoplasma gondii* and their capacity to reduce the dye nitroblue tetrazolium (NBT). Additional experiments were made in order to know if FI-PC24h could be originated from FI-PC2h.

2 Materials and methods

2.1 Animals and antigens

Wistar adult rats weighing 200–300 g were used. Saline extract of RAG from syngeneic rats were prepared as described in the work of Yantorno et al. [12]. MRAG were prepared by coupling their proteins with diazonium derivates of arsanilic and sulfanilic acid following a procedure described previously [13]. Purified fractions of RAG were obtained by chromatography on Sephadex G-100. Fraction I (FI) contained the autoantigenic macromolecules [14].

2.2 Peritoneal cells (PC)

The PC were obtained from normal rats (N-PC) or rats that had been injected 2 h or 24 h previously with 200 μg/0.5 ml of RAG (FI-PC2h and FI-PC24h). The animals were bled under ether anesthesia, the peritoneal cavity was opened, and the cells were collected in HBSS. The cell suspension was centrifuged at 800 × g for 5 min, washed twice in HBSS, once in RPMI and then diluted to obtain the appropriate concentration.

2.3 Microorganisms

C. albicans (no. 102/85 strain) was obtained from the stock culture collection of the Universidad Nacional de Córdoba. Cultures were grown on Sabouraud's glucose agar for 3 days at 37 °C and maintained by weekly subculture in the same medium. The RH strain of *T. gondii* maintained by regular i.p. passage in mice was used. Parasites were harvested by washing out the peritoneal cavity with PBS, pH 7.2. Exudate was collected on the third day of infection. The parasites were washed with PBS, counted and immediately used for the assays.

2.4 Phagocytosis assay

For the phagocytosis assay 0.1 ml of PC suspension containing 1×10^6 cells was combined with 0.1 ml of normal rat serum and 0.1 ml containing 1×10^7 yeast or parasites. The mixture was centrifuged at 100 × g for 15 min and incubated at 37 °C in an atmosphere of 5% CO_2 for 60 min. After resuspension, the cells were washed twice with HBSS and stained with Giemsa. Two hundred cells were assessed to determine the percentage of phagocytes (percentage of MΦ which had engulfed one or more yeast or parasite cells).

2.5 NBT assay

For the NBT assay 0.1 ml of PC suspension containing 1×10^6 cells was combined with 0.1 ml of normal rat serum, 0.1 ml of suspension containing 1×10^7 yeast and 0.1 ml of 0.2% NBT. The mixture was centrifuged at 100 × g for 15 min and incubated at 37 °C in an atmosphere of 5% CO_2 for 60 min. After that, the cells were fixed and stained with 10% Giemsa and the percentage of positive cells (those with blue formazan particles within the cytoplasm) was counted.

2.6 Evaluation of cell surface markers

An indirect immunofluorescence test using mAb directed against OX41, OX6 and OX17 surface markers (kindly supplied by W. F. Hickey) was performed following the procedures described by Hickey et al. [15]. In brief, PC were suspended in RPMI 1640 at a final concentration of 1×10^6 cells/ml. Three hundred microliters of this cell suspension was centrifugated and the cell pellet obtained was mixed with 100 μl of an appropriated dilution of mAb, mixed and incubated at 4 °C for 60 min. The cells were collected by centrifugation and washed twice with HBSS containing 0.02% sodium azide. After the second wash the cell pellets were resuspended in 15 μl of an 1 : 4 dilution of FITC-conjugated rabbit anti-mouse IgG and then incubated for 60 min at 4 °C. The samples were again washed twice in HBSS containing sodium azide. A minimum of 200 cells were counted by ultraviolet microscopy to determine the percentage of positive cells.

2.7 Treatment of FI-PC2h

In some experiments FI-PC2h were cultured during 22 h in RPMI 1640 supplemented with 10% of normal rat serum at 37 °C in an atmosphere of 5% CO_2 (FI-PC2-24h*in vitro*), while in other experiments 10^7 FI-PC2h were injected i.p. in syngeneic recipients and 22 h later their PC were harvested by washing out the peritoneal cavity (FI-PC2-24h*in vivo*).

2.8 Transfer of FI-PC2-24h *in vivo* and FI-PC2-24h *in vitro*

Two groups of rats (RFI-PC2-24h*in vitro* and RFI-PC2-24h*in vivo*) received 1 ml of cellular suspension containing 1×10^7 FI-PC2-24h*in vitro* or FI-PC2-24h*in vivo*, respectively, on days −10 and −3. The response controls (RN-PC) received 1×10^7 N-PC. As suppression controls we used groups of rats injected i.p. with 1×10^7 FI-PC2h on days −10 and −3 and as potentiation controls we used another group of rats injected i.p. with 1×10^7 FI-PC24h on the same days. Rats of all groups were immunized i.d. with 5 mg of MRAG on day 0. The antigenic material in a volume

0.5 ml was emulsifield in an equal volume of CFA. The animals were challenged in the footpad on day 15.

2.9 DTH

The footpad test was accomplished by injecting 0.1 mg of MRAG dissolved in 0.1 ml of 0.15 M NaCl into the right rear footpad and an equal volume of 0.15 M NaCl into the left footpad. The swelling was observed and measured with a micrometer 24 h after injection and the increase was determined by the following formula:

$$Z \times 10 = C$$

where Z is the difference in footpad swellling between the right and the left footpads and a C value of 4×10^{-1} mm or higher was considered to be a positive footpad test.

2.10 Irradiation and BM reconstitution

Two groups of rats, FI-PC24h(IR) and FI-PC24h(IR-BM), were irradiated (850 rad) from a ^{137}Cs source. Fifteen to twenty hours later, the FI-PC24h(IR-BM) group was reconstituted i.v. with 40×10^7 syngeneic rat BM cells (harvested from femurs and tibiae). Both groups of rats were injected i.p. with 200 μg of FI of RAG and 10^7 N-PC 6 days after irradiation and 24 h later their PC were harvested by washing out the peritoneal cavity.

2.11 Statistics

The Mann-Whitney U non parametric test was used for comparison of differences between groups. Each group consisted of a minimum of five animals. Values of $p < 0.001$ were considered as significant.

3 Results

3.1 Phagocytic activity against *C. albicans* and capacity to reduce NBT

To gain further knowledge of the characteristics of the two populations of PC with opposite immunologic effects on the autoimmune response to autoantigens of RAG, the phagocytic activity of the FI-PC2h, FI-PC24h and N-PC were assessed.

The phagocytosis of *C. albicans* showed that FI-PC2h had a similar phagocytic activity to that found in N-PC. In contrast, the phagocytic activity of FI-PC24h was significantly diminished compared with the N-PC (Fig. 1). The O_2^- production by PC under study was analyzed by incorporating the NBT dye in to the phagocytosis assay of *C. albicans*. Fig. 1 shows that the FI-PC2h ability to reduce the NBT dye was significantly elevated compared to N-PC. In contrast, the capacity of FI-PC24h to reduce the dye was significantly diminished.

3.2 Behavior of FI-PC2h and FIPC24h towards *T. gondii*

In view of the different phagocytic activity against *C. albicans* of the PC under study, it was of interest to know the behavior of FI-PC2h and FI-PC24h towards an obligate intracellular parasite as *T. gondii*. In the first part of the experiment the PC were incubated with the parasite for 1 h. After the incubation, the PC without acidophilic granules, which constitute the predominant populations of N-PC and FI-PC2h (N-PC: 66%; FI-PC2h: 70%; FI-PC24h: 28%; [11]), were mainly invaded by *T. gondii* (6–12 parasites/cell and marked signs of cellular alterations). The cells with acidophilic granules, which constitute the predominant population of FI-PC24h [11], rarely presented *T. gondii* in their cytoplasma (only one–two parasites/cell inside the refringent vacuole). In fact, the percentage of PC with *T. gondii* inside the cytoplasma was 63.4 ± 2.4% in N-PC, 69.8 ± 5.0% in FI-PC2h and 31.8 ± 4.8% in FI-PC24h (Fig. 2).

In a second experiment the PC were incubated with *T. gondii* for 4 h. A marked reduction in the number of N-PC and FI-PC2h was observed after incubation when compared with the number of cells at time zero (% of reduction: 66.3 ± 4.1 and 68.1 ± 2.9, respectively; Fig. 3). In contrast, a slight reduction in the number of FI-PC24h was found after incubation (28.6 ± 3.7%). The remainder N-PC, FI-PC2h and FI-PC24h showed acidophilic granules and only a very low percentage presented one or two parasites inside the refringent vacuoles.

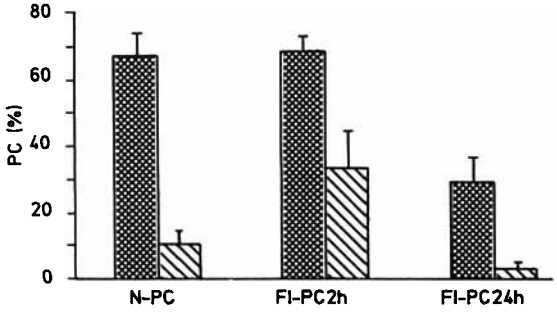

Figure 1. Percentage of PC from normal rats (N-PC) or rats injected i.p. 2 h or 24 h previously with 200 μg/0.5 ml of FI of RAG (FI-PC2h and FI-PC24h, respectively) with phagocytic activity against *C. albicans* (■) or capacity to reduce NBT (▨) (n = 6 for each group).

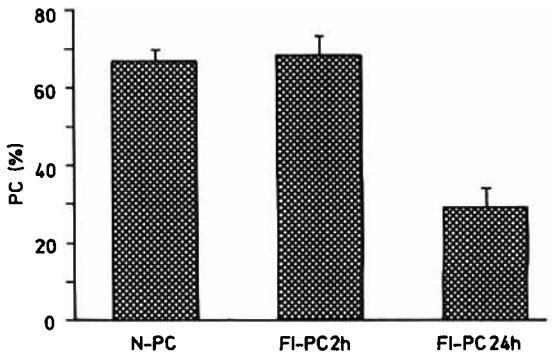

Figure 2. Percentage of N-PC, FI-PC2h and FI-PC24h with *T. gondii* inside the cytoplasma 1 h after incubation with the parasite (n = 6 for each group).

3.3 Phenotypic and functional characteristics of FI-PC2-24h *in vivo* and FI-PC2-24h *in vitro*

3.3.1 General remarks

To find out if FI-PC24h could originate from FI-PC2h, the FI-PC2h were either *in vitro* cultured for 22 h (FI-PC2-24h *in vitro*) or injected i.p. in syngeneic recipients and 22 h later their PC were harvested (FI-PC2-24h *in vivo*). The characteristics of both FI-PC2-24h were studied through the analysis of the surface markers and their effect on the autoimmune response.

3.3.2 Surface markers

The surface markers of the different PC were analyzed by the indirect immunofluorescence technique using mAb OX41, OX17 and OX6 surface markers (Fig. 4). Most PC in all the groups showed the OX41 MΦ surface marker (mean: 75.6 ± 4.6%). The N-PC showed a low percentage of OX6 and OX17 markers (14.2 ± 3.8% and 15.2 ± 5.5%, respectively). The OX17 marker was significantly increased in both FI-PC2-24 *in vitro* and FI-PC2-24h *in vivo* (OX17 : 44.1 ± 1.0 and 31.7 ± 12.2) whereas the OX6 marker was expressed by 14.3 ± 3.4 and 6.9 ± 2.0 of FI-PC2-24 *in vitro* and FI-PC2-24h *in vivo*, respectively. A selective increase of OX17 surface marker was also observed in FI-PC2h (OX6 : 13.5 ± 1.6 and OX17 : 53.6 ± 12.5%) whereas the FI-PC24h showed a significant increase of OX6 marker (OX6 : 67.7 ± 10.7 and OX17 : 14.5 ± 3.4%) when compared with N-PC.

3.3.3 Effect on the autoimmune response against MRAG

The effect of FI-PC2-24h *in vitro* and FI-PC2-24h *in vivo* on the autoimmune response was studied by administering such cells to recipient normal rats on days − 10 and − 3. The experimental recipients (RFI-PC2-24h *in vitro* and RFI-PC2-24h *in vivo*), the response (RN-PC), the suppression (RFI-PC2h) and the potentiated (RFI-PC24h) control recipients were immunized with MRAG in CFA on day 0. The DTH against MRAG was analyzed 15 days after the immunization.

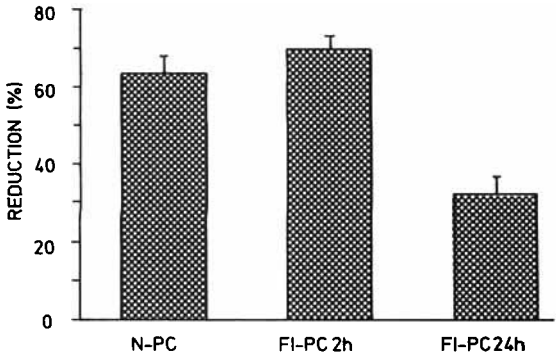

Figure 3. Percentage of reduction of N-PC, FI-PC2h and FI-PC24h number 4 h after incubation with *T. gondii* (n = 6 for each group).

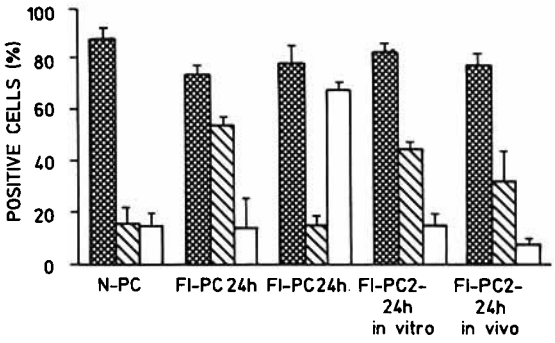

Figure 4. Percentage of OX41$^+$ (▩) OX17$^+$ (▨) and OX6$^+$ (□) PC from N-PC, FI-PC2h FI-PC24h, PC from rats injected i.p. 22 h previously with FI-PC2h (FI-PC2-24h *in vivo*) and FI-PC2h cultured during 22 h (FI-PC2-24h *in vitro*) (n = 6 for each group).

The DTH response against MRAG in RFI-PC2h, RFI-PC2-24h *in vitro* and RFI-PC2-24h *in vivo* was significantly suppressed compared to the response found with RN-PC (Fig. 5). In contrast, the DTH response against MRAG in RFI-PC24h was significantly increased with respect to that observed in RN-PC animals.

3.4 Effect of the irradiation and BM reconstitution on the ability to originate I-A$^+$ PC

In view of the fact that FI-PC24h could not derive from FI-PC2h in the above experiments we studied the effect of the irradiation and BM reconstitution on the ability of N-PC to yield I-A$^+$ PC through the analysis of the surface markers of PC obtained from irradiated and irradiated and BM-reconstituted rats.

The PC obtained 24 h after injection of FI-PC24h(IR) rats with N-PC plus FI showed a lack of ability to yield I-A$^+$ PC (Fig. 6). They showed a significant increase of the OX17 surface marker (OX6 : 17.6 ± 1.5%; OX17 : 41.9 ± 2.5%) when compared with N-PC (Fig. 4). In contrast, an

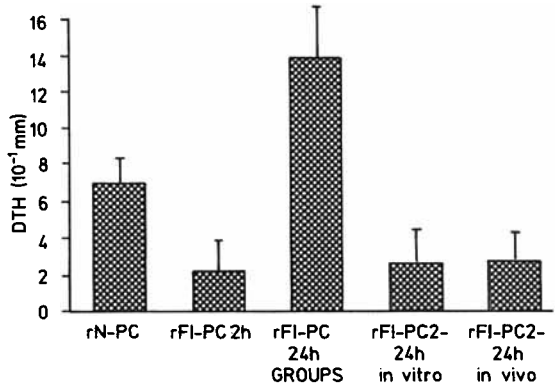

Figure 5. DTH response to MRAG studied in recipients 15 days after immunization. On days − 10 and − 3 the animals were injected with normal PC (RN-PC), PC from rats injected i.p. 2 h or 24 h previously with 200 μg/0.5 ml of FI of RAG (RFI-PC2h or FI-PC24h, respectively), PC from rats i.p. injected 22 h previously with FI-PC2h (RFI-PC2-24h*in vivo*) or FI-PC2h cultured during 22 h (RFI-PC2-24h*in vitro*). All groups of animals were immunized with MRAG in CFA on day 0 (n = 6 for each group).

enhancement of I-A$^+$ PC could be observed when the PC were obtained from FI-PC24h(IR-BM) rats 24 h after injection of N-PC plus FI. These PC showed a significant increase of OX6 surface marker (OX6: 36.8% ± 3.4%; OX17: 17.4 ± 2.8%) when compared with N-PC. It is important to note that the selective enhancement of the OX17 surface marker in PC from FI-PC24h(IR) rats and of the OX6 surface marker in PC from FI-PC24h(IR-BM) rats was previously found in FI-PC2h and FI-PC24h, respectively (Fig. 4).

4 Discussion

A state of specific tolerance to MRAG is induced when low doses of purified fractions of RAG are injected i.p. in the soluble form before the immunization with MRAG-CFA [7]. The unresponsiveness is specific for the autoantigen at both the cellular and humoral level. Adoptive transfer experiments have shown that MRAG-specific, CY-sensitive, inducer-phase T_s cells, capable of suppressing the DTH reaction but not the antibody response, are involved [8, 9]. In attempting to analyze the requiriements for induction of these MRAG-specific T_s cells we recently demonstrated that this cell population can also be induced by the injection of PC-coupled with antigen *in vitro*, prior to the immunization [10]. It was also demonstrated that FI-PC2h were involved in the inducer-phase T_s cell induction, while FI-PC24h were involved in the induction of cells responsible for the autoimmune response [11].

It has been suggested that the accessory cells involved in T_h cells and T_s cells induction are different [16]. Most notably, resistance to ultraviolet irradiation has been used to functionally distinguish these APC populations [17]. A few reports have claimed that the MΦ involved in T_s cells induction are sensitive to treatment with low doses of CY while the APC controlling induction of T_h cells are CY resistant [18]. However, another report claims that the MΦ responsible for induction of T_s cells are CY resistant [19]. A clear functional distinction between the APC responsible for T_h and those responsible for T_s cell induction is not available.

We show in this work that both PC have functionally different characteristics. In fact, the FI-PC2h have an elevated phagocytic activity against *C. albicans* and an increased capacity to reduce the NBT dye, while FI-PC24h have a diminished capacity to reduce the dye and a disminished phagocytic activity. Besides, the different action of *T. gondii* on both populations appears to indicate that the parasite can invade FI-PC2h while FI-PC24h offer resistance to *T. gondii* aggression at least during the time of incubation in these experiments. These results are in agreement with those found by several authors in different systems that involve heteroantigens [6, 20]. In fact, Noma et al. [6] have demonstrated in the NP system that the adherent population responsible for induction of T_s cells expresses Ia and Mac-1 determinants but lacks conventional lymphocyte markers, including CD5 and Ig. Furthermore, the APC that induce T_s cells response are contained within the phagocytic FcR-bearing fraction. In contrast, the nonphagocytic, FcR$^-$, nonspecific esterase-negative dendritic fraction is unable to induce T_s activity.

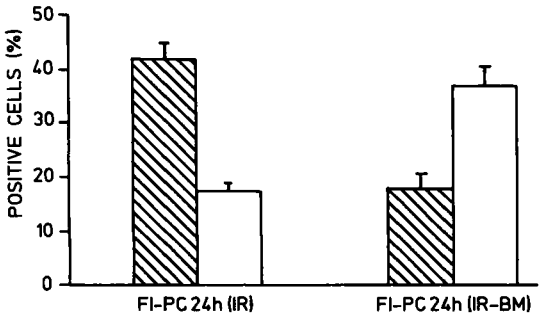

Figure 6. Percentage of OX17$^+$ (▨) and OX6$^+$ (□) of PC from FI-PC24h(IR) and FI-PC24h(IR-BM) rats injected 24 h previously with 200 μg of FI and 1 × 10^7 N-PC.

Another remarkable difference between the PC under study was the MHC molecules present on their surface. In fact, it was previously reported that the the I-E surface marker predominated in FI-PC2h and that the ability of these cells to induce antigen-specific, inducer-phase T_s cells could be blocked by treating these PC with anti-I-E antibody. In contrast, the FI-PC24h showed an increase of the I-A surface marker and their capacity to induce potentiation of the autoimmune response to autoantigens of RAG could be blocked by the treatment of FI-PC24h with anti-I-A antibody. In this work, the analysis of the surface markers and the effect on the autoimmune response of FI-PC2-24h*in vivo* and FI-PC2-24h*in vitro* appear to indicate that the population obtained 24 h after injection of the FI of RAG could not originate from the population present 2 h after injection of the FI of RAG. Moreover, the ability of resident PC to yield I-A$^+$ FI-PC24h is abrogated when MΦ and their precursors are eliminated by irradiation of rats prior to injection of N-PC plus FI of RAG and restored by BM reconstitution of irradiated rats. The BM origin of Ia$^+$ MΦ in the peritoneal cavity was previously reported by Scher et al. [21]. The results obtained in this experimental model of autoimmunity appear to indicate that the enhancement of I-A$^+$, but not I-E$^+$ cells after injection of FI into the peritoneal cavity would need, at least in part, BM precursors.

The H-2 haplotype of the APC used to educate T cells ultimately controls the restriction specificity of both T_h cells and T_s cells populations [22]. If the results from our model system can be generalized, they imply that presentation of an antigen by a particular MΦ (subset/differentiation state) dictates the final outcome of the immune response (immunity or suppression). In the case of FI-PC2h, most of them being I-E$^+$, there is predominant generation of T_s cells, thus tilting the balance of immunity toward suppression whereas FI-PC24h, most of them being I-A$^+$, selectively induce immunity. The concept that I-E-controlled responses might be particularly important in induction of specific suppression of autoreactive lymphocytes, while leaving the remainder of the immune system intact, would be useful to the knowledge and therapy of autoimmune diseases.

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