

A monoclonal antibody to the rat Crry/p65 antigen, a complement regulatory membrane protein, stimulates adhesion and proliferation of thymocytes

N. ARSENOVIĆ-RANIN,* D. VUČEVIĆ,† N. OKADA,‡ M. DIMITRIJEVIĆ* & M. ČOLIĆ†
*University School of Pharmacy, Belgrade, Yugoslavia, †Institute of Medical Research, Military Medical Academy, Belgrade, Yugoslavia, and ‡Department of Molecular Biology, Nagoya City University School of Medicine, Nagoya, Japan

SUMMARY

A murine monoclonal antibody (mAb), 3F10, was produced by fusion of spleen cells obtained from mice immunized with a rat cortical thymic epithelial cell line (R-TNC.1) stimulated with interferon- γ and P3X myeloma cells. 3F10 recognized an antigen expressed both on thymocytes and non-lymphoid cells in the thymus. Flow cytometry showed that 3F10 stained more than 98% thymocytes and 90% R-TNC.1 cells. Immunoprecipitation and Western blot studies demonstrated that 3F10 reacted with molecules of 55 000 and 65 000 MW from both thymocyte and R-TNC.1 cell lysates. 3F10 recognized the same antigen on Chinese hamster ovary cells transfected with rat Crry as did 5I2 mAb, confirming the specificity of 3F10 mAb for the rat homologue of mouse Crry/p65, a membrane-bound complement regulatory protein. 3F10 mAb induced homotypic aggregation of thymocytes and exhibited an additive effect on the aggregation evoked by phorbol myristate acetate. The aggregation was dependent on active cell metabolism, intact cytoskeleton, divalent cations and activation of protein phosphatases 1 and 2A (as assessed by use of okadaic acid). In contrast, H-7, HA1004 and genistein partially inhibited, whereas staurosporine potentiated the aggregation of thymocytes triggered by 3F10. 3F10 mAb also stimulated binding of thymocytes to the R-TNC.1 line. Both homotypic and heterotypic adhesive interactions are mediated by leucocyte function-associated antigen-1 (LFA-1). In addition, 3F10 stimulated proliferation of thymocytes induced by suboptimal concentrations of concanavalin A. These data suggest that rat Crry/p65 might be involved in the regulation of both cell adhesion and activation of thymocytes. This is a novel, non-complement-dependent function of Crry/p65.

INTRODUCTION

The thymus has an important role in the generation of T cells. It provides a microenvironment for a complex series of steps in intrathymic T-cell development: the attraction of precursors, commitment to the T-cell lineage, induction of the T-cell receptor gene rearrangement, accessory molecule expression, repertoire expansion, major histocompatibility complex (MHC) molecule-based selection (positive and negative), acquisition of functional maturity and migratory capacity.¹ This maturation process involves bidirectional interactions between developmental thymocytes and different components of the thymic microenvironment, such as epithelial cells, dendritic cells, macrophages, fibrous stroma, and extracellular matrix.^{2,3} Interactions involve direct cell–cell contacts and

soluble mediators (cytokines, thymic hormones and other biologically active substances). Numerous studies suggested that direct contacts between thymic epithelial cells (TEC) and developing thymocytes are mediated by specific cell-surface interactions such as CD2/leucocyte function-associated antigen (LFA)-3, LFA-1/intracellular adhesion molecule (ICAM)-1, class I MHC/CD8, class II MHC/CD4, Thy-1, very late antigen (VLA)-4 and several newly discovered adhesion molecule (reviewed in 4).

A recent approach to the analysis of these intrathymic mechanisms has been to raise monoclonal antibodies (mAb) to molecules expressed by TEC.⁵ Unexpectedly, some of the mAb raised against TEC recognize antigenic determinants shared between TEC and developing thymocytes.^{6–10} Detailed studies have confirmed that usually both cell types synthesize the molecules *de novo* and that the antigen detected on the two populations is genuinely the same, rather than simply sharing a cross-reactive epitope.^{6,7,9} The significance of molecules shared between two interacting cells in the thymus is not clear. They could be involved in homotypic and heterotypic binding with the same or a complementary

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Correspondence: Dr M. Čolić, Institute of Medical Research, MMA, Crnotravska 17, Belgrade, Yugoslavia.

structure on the opposing cell surface, respectively, or serve as receptors for soluble ligands.^{2,3}

We have recently produced a mAb, named 3F10, which recognizes one such shared antigen expressed both on thymocytes and non-lymphoid cells in the rat thymus. We report here that it is specific for the rat Crry/p65 antigen, a complement regulatory membrane protein. The mAb stimulates homotypic aggregation of thymocytes and proliferation of these cells to suboptimal concentrations of concanavalin A (Con A). In addition, it increases heterotypic binding of thymocytes to a rat TEC line *in vitro*. Both adhesive interactions are LFA-1 dependent. This is a novel role of rat Crry/p65, not recognized so far.

MATERIALS AND METHODS

Monoclonal antibodies and reagents

3F10 mAb was produced at the Institute of Medical Research (MMA), Belgrade, Yugoslavia. Briefly, fusion was performed between P3X myeloma cells and splenocytes from mice immunized with a rat cortical TEC line (R-TNC.1) stimulated with interferon- γ (IFN- γ). This mAb (immunoglobulin G1, IgG1) was purified by salt fractionation. Biotinylation of the mAb was performed using *N*-hydroxysuccinimido (NHS)-biotin (Sigma, St Louis, MO). 5I2 mAb (IgG1) reactive with the rat Crry molecule was produced at the Department of Molecular Biology, Nagoya City University School of Medicine, Nagoya, Japan by Takizawa *et al.*¹¹ WT.3 (anti-rat CD18; IgG1), WT.1 (anti-rat CD11a; IgG2a) and 1A29 (anti-rat CD54; IgG1) were produced at the Department of Immunology, Metropolitan Institute of Medical Science, Tokyo, Japan¹² and were kindly donated by Prof. M. Miyasaka, Osaka, Japan. R73 (anti-rat $\alpha\beta$ T-cell receptor, TCR) and W3/25 (anti-rat CD4) mAb were purchased from Serotec, Oxford, UK. A peroxidase-conjugated rabbit anti-mouse immunoglobulin was purchased from Dako, Denmark, whereas a sample of fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse immunoglobulin was obtained from Inep, Zemun, Yugoslavia.

The reagents used were: phorbol myristate acetate (PMA; Sigma), sodium azide (Merck, Darmstadt, FRG), cytochalasin B (Sigma), cycloheximide (Sigma), 1-(5-isoquinolinesulphonyl)-3-methyl-piperazine (H-7; Sigma), *N*-(6-aminohexyl)-1-naphthalenesulphonamide (W-7; Sigma), *N*-(2-guanidinoethyl)-5-isoquinolinesulphonamide (HA1004; Sigma), genistein (ICN, Cleveland, OH), okadaic acid (Sigma), wortmannin (Sigma), staurosporine (Sigma), bisindolylmaleimide (Calbiochem, San Diego, CA), Con A (Sigma)

Cells and cell lines

AO rats, male, 6–8-week-old, bred at the Farm for Experimental Animals, MMA (Belgrade, Yugoslavia), were used as a source of thymocytes. Thymocytes were isolated from thymuses by teasing the organs against a stell mesh to obtain a single-cell suspension. The R-TNC.1 line was established at the Institute of Medical Research, MMA (Belgrade, Yugoslavia). This line was characterized as a type of rat cortical TEC with the nursing activity.¹³ It was propagated in RPMI-1640 medium (Serva, Heidelberg, Germany) containing 15% fetal calf serum (FCS; Flow Laboratories, Irvine, UK), 5 μ g/ml insulin (Serva), 50 nm dexamethasone (ICN-Galenika,

Belgrade, Yugoslavia) and 10 ng/ml epidermal growth factor (EGF; Serva). The coding region for the rat 5I2 antigen was from the cDNA plasmid clone 18.¹⁴ The 5I2 antigen cDNA was subcloned into *SalI* and *SpeI* sites (sense strand vector) or *SacI* and *KpnI* sites (antisense strand vector) of the eukaryotic expression vector pBK-cytomegalovirus (CMV) (Stratagene, La Jolla, CA). The sense strand vector was removed from the *NheI* to *SpeI* region (the region containing the lac promoter and lacZ ATG). Both vectors were digested by *NaeI* to linearize before transfection. The Chinese hamster ovary (CHO) cell line was transfected by electroporation using Gene Pulser (Bio-Rad Laboratories, Hercules, CA).

Flow cytometry

Thymocytes (1×10^6 /tube) and R-TNC.1 cells (2×10^5 /tube) were incubated with 5 μ g/ml 3F10 mAb in phosphate-buffered saline (PBS) containing 0.1% sodium azide and 2% FCS (PBS/FCS) for 30 min at 4°, washed twice with PBS/FCS, and stained with FITC-conjugated goat anti-mouse IgG for 30 min at 4°. Double immunofluorescence was used to determine the expression of 3F10 on different thymocyte subsets. For this purpose thymocytes were incubated with saturating concentrations of mAbs at the following order: R73 mAb, anti-mouse immunoglobulin FITC, 3F10 biotinylated antibody, streptavidin-phycoerythrin (PE). After the final washing with PBS/FCS and PBS, respectively, cells were analysed on an EPICS XL-MLC cytofluorometer (Coulter, Krefeld, Germany) using single- or double-colour immunofluorescence. In the experiments using double staining the expression of 3F10 was determined on $\alpha\beta$ TCR⁻, $\alpha\beta$ TCR^{lo} and $\alpha\beta$ TCR^{hi} thymocyte subsets. 5I2 antigen sense strand cDNA transfected or control (antisense strand transfected) CHO cells were suspended by incubating with 0.02% ethylenediamine tetra-acetic acid (EDTA)/PBS for 10 min at 37°. Cell suspensions containing 1×10^6 cells were incubated with 10 mg/ml goat IgG (Chemicon, Temecula, CA) for 10 min at room temperature to block non-specific binding. Then 3F10 mAb or 5I2 mAb were added to cells at final concentration 20 μ g/ml and incubated for 30 min on ice. The suspensions were centrifuged and washed three times with cold PBS, followed by incubation with FITC-conjugated goat anti-mouse immunoglobulin (Becton Dickinson, Mountain View, CA) for 30 min on ice and washed. Finally, cells were analysed by fluorescence-activated cell sorting on a FACSCulibur (Becton Dickinson). Dead cells were excluded using staining with 2 mg/ml propidium iodide.

Immunoperoxidase staining

Cryostat sections of rat thymuses were fixed in acetone for 10 min, air dried and washed in Tris-buffered saline (TBS). The fixed sections were incubated for 30 min in methanol containing 0.3% H₂O₂ for inhibition of endogenous peroxidase activity. After washing in TBS, the sections were incubated with 3F10 mAb for 30 min, washed, and incubated 30 min with goat anti-mouse IgG conjugated with peroxidase (Dako). Revelation of the peroxidase activity was demonstrated by 0.06% diaminobenzidine (DAB; Serva) and 0.01% H₂O₂. After washing, the sections were counterstained with haematoxylin prior to mounting, and were observed under a conventional light microscope.

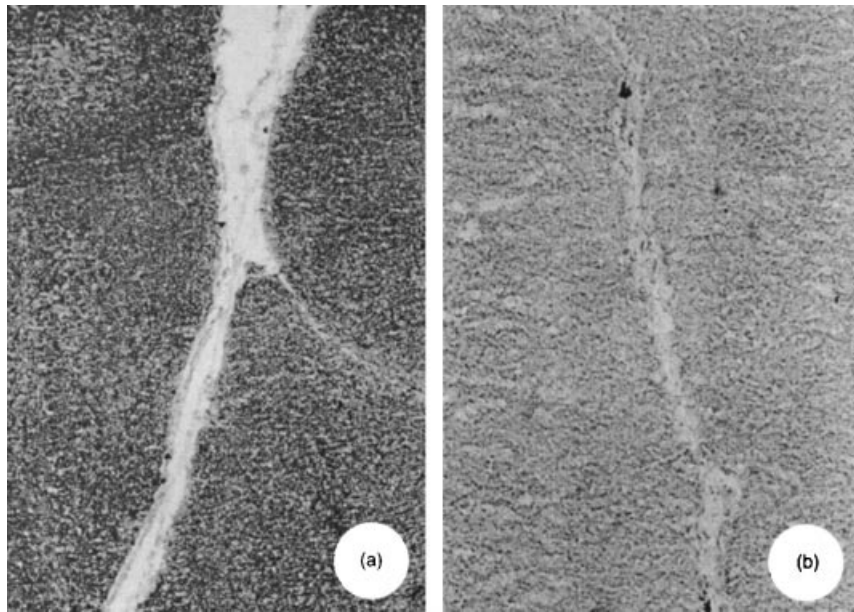


Figure 1. Immunoperoxidase staining of the rat thymus by 3F10 mAb (a) and secondary antibody alone (negative control) (b). 3F10 antigen is expressed on both thymocytes and stromal cells. Original magnifications: $\times 16$.

Western blotting

Trypsinized R-TNC.1 cells (0.1% trypsin in 0.02% EDTA) and thymocytes were washed three times in PBS. Cells (3×10^8 thymocytes or 1×10^7 R-TNC.1 cells) were lysed in 1 ml ice-cold lysis buffer containing 0.5% Nonidet P-40, 50 mM Tris (pH 7.6), 300 mM NaCl, and 10 $\mu\text{g/ml}$ of aprotinin, iodoacetamide, leupeptin and phenylmethylsulphonyl fluoride for 15 min on ice, centrifuged at 12000 g for 10 min, and soluble materials were diluted 1:1 with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer without 2-mercaptoethanol (2-ME). For Western blotting, the cell lysates were boiled for 3 min and run on 10% SDS-PAGE (30 μl per lane) transferred to polyvinylidene difluoride (PVDF) membranes and blotted with 3F10 mAb followed by a peroxidase-conjugated secondary antibody. Proteins were visualized by 0.5% DAB and 0.01% H_2O_2 . A mixture of standard protein markers (Sigma) was used for the determination of relative molecular mass.

Immunoprecipitation

Thymocytes ($3 \times 10^7/\text{ml}$) in cold RPMI-1640 medium (pH 8.0) were incubated 40 min at 4° with 0.2 mg/ml normal human serum (NHS)-biotin, washed three times in RPMI-1640 pH 7.2–7.4, and lysed in 300 μl lysis buffer as described above. Biotinylated thymocyte lysate was incubated overnight at 4° with the indicated mAb adsorbed to protein A-sepharose. To avoid non-specific adsorption, lysates were precleared for 2 hr with protein A-sepharose. Immunoprecipitates were centrifuged at 12 000 g for 2 min, washed three times in lysis buffer containing 0.1% Nonidet P-40, resuspended in 80 μl SDS-PAGE sample buffer without 2-ME and resolved by electrophoresis on 10% PAGE. Immunoblot analysis was performed by transferring separated proteins onto the PVDF membrane. The membrane was then probed with 1:800 dilution of peroxidase-conjugated streptavidin. In experiments

in which the relationship between 3F10 and 5I2 antigen was studied, lysates of nonlabeled thymocytes were immunoprecipitated with 3F10 mAb, run on 10% PAGE, transferred onto PVDF membrane and then analysed by Western blotting with either 3F10 or 5I2 mAb.

Semiquantitative aggregation assay

To measure homotypic adhesion we used the modified method described by Rothlein and Springer.¹⁵ Thymocytes were resuspended in RPMI-1640 medium with 10% FCS at 5×10^6 cells/ml and placed in 96-well flat-bottom microtitre plates (ICN, Costa Mesa, CA). The cells were incubated at 37° in the absence or presence of mAbs and/or PMA. Inhibition experiments were done by preincubating cells with the indicated blocking mAb at 4° or with different reagents at 37° for 30 min. The plates were observed by indirect microscopy after various time periods. Scores ranged from 0 to 5: 0, no aggregation; 1, less than 10% of the cells in aggregates; 2, 10–50% of the cells formed small clusters; 3, 50–80% of the cells in small loose and/or compact clusters; 4, >80% of the cells formed large loose and/or compact clusters; 5, nearly 100% of the cells in large very compact aggregates. Results were scored by two independent observers.

Quantitative aggregation assay

Thymocytes were resuspended in RPMI-1640 with 10% FCS at $1 \times 10^7/\text{ml}$ and placed in 2-ml plastic tubes. Stimuli were added in a final volume of 200 $\mu\text{l}/\text{tube}$ and cells were incubated at 37° for 2 hr. Where indicated, cells were preincubated with the various mAbs at 4° or metabolic inhibitors at 37° for 30 min. After incubation cells were mildly vortexed and free cells were counted:

$\% \text{ cells in aggregates} = 100 \times [1 - (\text{number of free cells after incubation})/(\text{number of free cells in control before incubation})]$.

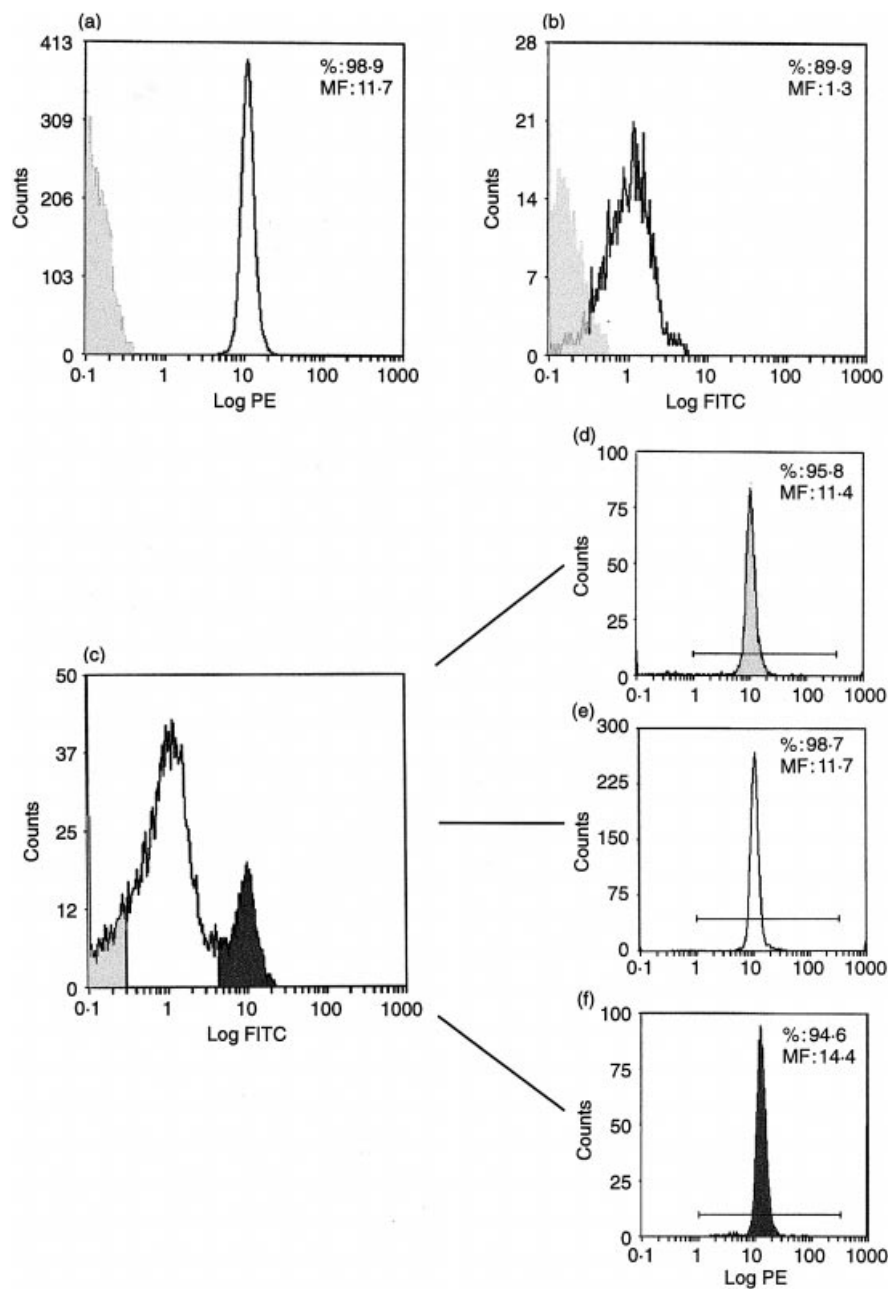


Figure 2. Surface expression of the 3F10 antigen on thymocytes (a) and R-TNC.1 cells (b). Cells in suspension were incubated with 3F10 biotinylated mAb followed by staining with PE (thymocytes) or 3F10 unconjugated mAb and then with FITC-conjugated goat anti-mouse IgG (R-TNC.1 cells) and then analysed on an EPICS XL-MCL cytofluorometer. Percentages of positive cells and MF intensities given on histograms were subtracted from negative controls (hatched areas). Expression of the 3F10 antigen on thymocyte subsets according to the expression of $\alpha\beta$ TCR. Cells were labelled with R73 and 3F10 mAb as described and processed for double immunofluorescence analysis. The histograms for the 3F10 antigen expression on $\alpha\beta$ TCR⁻ (d), $\alpha\beta$ TCR^{lo} (e) and $\alpha\beta$ TCR^{hi} (f) thymocyte subsets were determined according to the different staining of thymocytes by R73 mAb (c).

Controls were cells incubated in the same conditions but without stimuli.

Binding assay

Trypsinized R-TNC.1 cells were plated on 96-well flat-bottomed plates (Flow laboratories; 1×10^4 cells/well in 200 μ l of medium) and grown for 2 days until confluent

monolayers were obtained. For experiments, the enriched medium used for standard growth of these cells was replaced with RPMI-1640 medium containing 10% FCS. Resting adult thymocytes (5×10^5 cells/well) were added to the wells and spun on confluent monolayers (20 g, 1 min). After 45 min of incubation at 37°, the plates were spun upside down (100 g, 30 s) and quickly filled with medium to prevent drying and subsequent damage of cells. Thymocytes were detached from

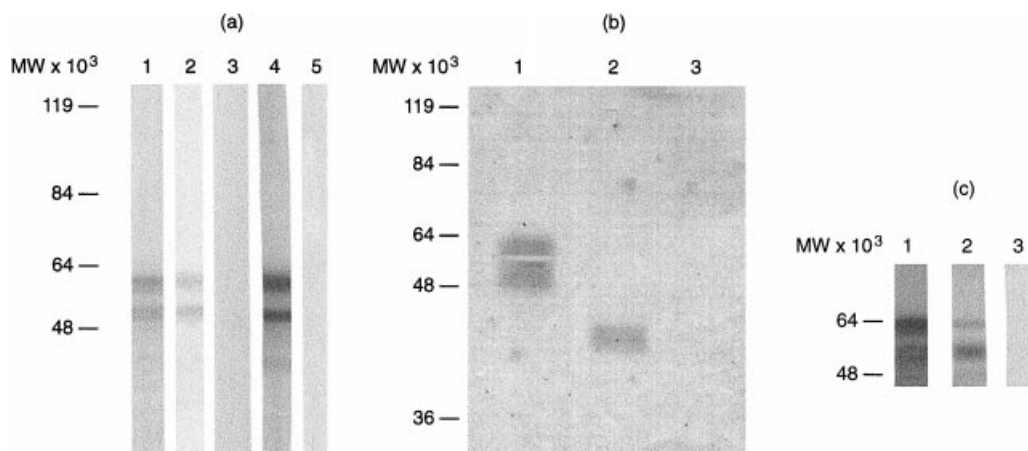


Figure 3. (a) Western blot of 3F10 antigen and 5I2 antigen from thymocyte and R-TNC.1 lysates. Lysates were resolved on 10% PAGE under non-reducing conditions, transferred to PVDF membrane, and blotted with 3F10 or 5I2 mAb (lane 1: thymocytes–3F10, lane 2: thymocytes–5I2, lane 3: thymocytes–negative control, lane 4: R-TNC.1–3F10, lane 5: R-TNC.1–negative control). (b) Immunoprecipitation of 3F10 antigen from the rat thymocyte lysate. Thymocytes were surface biotinylated, lysed in 0.5% NP-40 and soluble material was immunoprecipitated with 3F10 mAb (lane 1), positive control, anti-CD4 mAb (lane 2) or control IgG (lane 3), resolved on 10% PAGE under non-reducing conditions, transferred, and blotted with streptavidin–peroxidase. (c) Comparison of 3F10 and 5I2 mAb reactivity. Thymocyte lysate was immunoprecipitated with 3F10 mAb, resolved on 10% PAGE under non-reducing conditions, transferred to PVDF membrane, and analysed with 3F10 mAb (lane 1), 5I2 mAb (lane 2) or with secondary antibody alone (lane 3). Note the same reactivity of both mAb with the 65/55 000 MW heterodimer.

monolayers by pipetting. The antibody-mediated modulation of cell binding was assayed by preincubating thymocytes or R-TNC.1 cells with mAb for 30 min at 4°. All antibodies were continuously present during the assay. The number of attached cells was then counted and percentage binding was calculated as follows:

$$\% \text{ binding} = \frac{\text{number of attached thymocytes}}{\text{number of total thymocytes}} \times 100$$

The percentage of relative binding in the presence of specific or irrelevant mAb was calculated by comparing to corresponding controls (cells without mAb).

Proliferation assay

Thymocytes were cultivated in 96-well plates (1×10^6 /well) in the presence of 2.5 µg/ml or 0.625 µg/ml Con A without or with the addition of different concentrations of soluble 3F10 mAb or irrelevant mAb (IrmAb) (reactive with *Blas-tocystis hominis*, IgG1). Cells were cultured for 3 days, and [³H]thymidine was added during the last 18 hr of culture. Incorporated [³H]thymidine was measured as c.p.m. using a liquid scintillation counter (Beckman). Each test was done in triplicate.

RESULTS

Expression of the antigen recognized by 3F10 mAb in the thymus

3F10 mAb (IgG1) was recently established at the Institute of Medical Research, MMA, Belgrade, by fusing P3X myeloma cells and splenocytes from mice immunized with a rat cortical TEC line (R-TNC.1) stimulated with IFN-γ. The first aim of this study was to investigate binding of the 3F10 mAb on cryostat sections of the rat thymus using an immunoperoxidase method. Figure 1 demonstrates that both thymocytes and non-

lymphoid cells, including TEC, throughout the thymus were strongly positive. Flow cytometry showed membranous expression of the antigen by more than 98% thymocytes and more than 89% R-TNC.1 cells (Fig. 2). Double immunofluorescence demonstrated that all thymocyte subsets ($\alpha\beta$ TCR⁺, $\alpha\beta$ TCR^{lo} and $\alpha\beta$ TCR^{hi}) expressed the 3F10 antigen (Ag). However, based on the mean fluorescence (MF) intensity its expression on the $\alpha\beta$ TCR^{hi} thymocyte subset was higher compared to total thymocytes or other two cell subsets (Fig. 2).

Biochemical characterization of the 3F10 antigen

In order to define biochemical characteristics of the antigen recognized by 3F10, we analysed the lysates of thymocytes and R-TNC.1 cells by Western blotting and immunoprecipitation. Western blotting of both thymocyte and R-TNC.1 cell lysates using 3F10 mAb results in a dual band of 65 000 MW and 55 000 MW under non-reducing conditions (Fig. 3a). Under reducing conditions, 3F10 mAb did not react with the target molecules on the PVDF membrane (data not shown). Immunoprecipitation study of the thymocyte lysate also showed that 3F10 mAb immunoprecipitated molecules of approximately 65 000 and 55 000 MW (Fig. 3b). Recently, it has been demonstrated that a murine mAb, 5I2, specific for the rat homologue of the mouse Crry/p65 (a regulator of complement activation) immunoprecipitated a molecular complex from the rat erythrocyte lysate of the same biochemical characteristics as 3F10 mAb.¹¹ We showed that 5I2 mAb also recognized the 65/55 heterodimer in Western blot of the thymocyte lysate (Fig. 3a). In order to clarify whether these mAbs recognize the same antigens, the thymocyte lysate was immunoprecipitated with 3F10 mAb, resolved on 10% PAGE, transferred onto the nitrocellulose membrane and stained with 3F10 and 5I2 mAb. Results presented in Fig. 3(c)

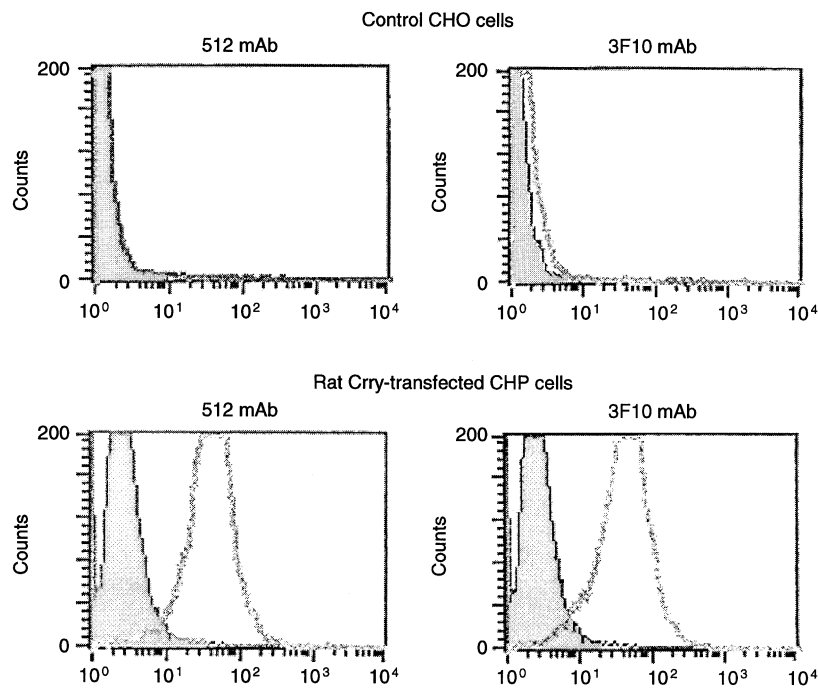


Figure 4. Reactivity of 3F10 and 5I2 mAb with rat Crry transfected CHO cells. CHO cells transfected with rat Crry as well as control CHO cells were incubated with 3F10 or 5I2 mAb followed by FITC-conjugated goat anti-mouse immunoglobulin. Cells were then analysed using FACSCulibur (Becton Dickinson). Note that both mAb stain Crry-transfected, but not control CHO cells. Hatched areas represent corresponding negative controls.

clearly demonstrate that 5I2 and 3F10 showed the same specificity.

3F10 mAb binds to CHO cells transfected with the rat Crry/p65

The final evidence for the specificity of 3F10 was obtained by testing its reactivity with the rat Crry-transfected CHO cells by flow cytometry. As shown in Fig. 4, both 3F10 and 5I2 mAb equally stained CHO-transfected, but not control CHO cells, confirming again the specificity of 3F10 mAb for the rat Crry/p65 antigen.

3F10 promotes homotypic adhesion of rat thymocytes

Initial experiments showed that 3F10 mAb was able to induce homotypic aggregation of rat thymocytes. Relatively weak, but still visible, aggregation was also observed using 5I2 mAb (data not shown). To explore this phenomenon in more detail, we compared thymocyte aggregation induced by 3F10 and PMA. Results presented in Fig. 5 show that 3F10 induced moderate homotypic aggregation of thymocytes (maximum score aggregation 2 after 2 hr). Similar or a bit stronger aggregation was observed using PMA whereas both agents had additive effects. Thymocyte aggregation was optimal using 25 µg/ml 3F10 and was still visible at the concentration as low as 0.5 µg/ml (data not shown).

Requirements for the 3F10-induced homotypic adhesion

The next experiments were designed to study the physiologic

requirements for the 3F10-induced homotypic aggregation. The results are presented in Table 1. 3F10 did not induce aggregation of thymocytes in HBSS medium without divalent cations (Ca^{2+} and Mg^{2+}) or with Ca^{2+} only, while the aggregation was restored in HBSS with Mg^{2+} in a dose-dependent manner. Similarly, the PMA-evoked aggregation required only Mg^{2+} , but neither Mg^{2+} concentrations used (1–5 mM) did not restore adhesion to the level seen in complete medium containing 10% FCS. Addition of Ca^{2+} in Hank's balanced salt solution (HBSS) with Mg^{2+} significantly increased both PMA and 3F10-evoked aggregation. The 3F10-induced aggregation was fully prevented by incubating cells at 4° or in the presence of sodium azide, confirming its dependence upon active cell metabolism and actin cytoskeleton (Table 1). Cycloheximide, an inhibitor of protein synthesis, had no effect on the aggregation. To determine the intracellular signalling pathways involved in the 3F10-induced aggregation, we preincubated thymocytes with different concentrations of H-7 (an inhibitor of protein kinase A (PKA), PKC and PKG), HA1004 (an inhibitor of PKA and PKG), staurosporine (a non-selective PK inhibitor), bisindolylmaleimide (a specific PKC inhibitor), genistein (an inhibitor of protein tyrosine kinases), okadaic acid (an inhibitor of protein phosphatases (PP)1 and 2A), W-7 (a blocker of calmodulin-dependent protein kinase) and wortmannin (an inhibitor of phosphatidylinositol 3-kinase). Table 1 shows that homotypic adhesion of thymocytes triggered by 3F10 mAb was partially suppressed by H-7, HA1004 and genistein and completely inhibited by okadaic acid. The adhesion was not modified by using bisindolylmaleimide, W-7 and wortmannin. Staurosporine potentiated the 3F10-induced homotypic aggregation of

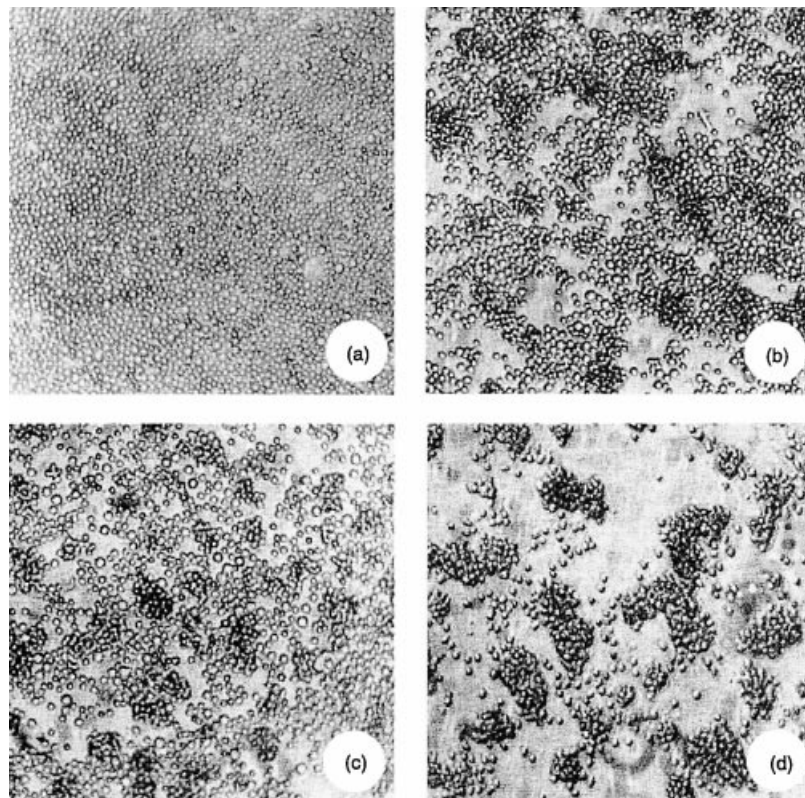


Figure 5. Induction of the thymocyte aggregation by 3F10, PMA or their combination. (1) Medium; (2) 3F10 mAb; (3) PMA; (4) PMA + 3F10 mAb. Cells were plated in 96-well plates (at 1×10^6 /well) with addition of 3F10 (25 $\mu\text{g/ml}$), PMA (50 ng/ml) or their combinations, incubated at 37° and photographed after 2 hr of culture. Original magnification: $\times 25$.

thymocytes. These results indicate that activation of PKC and/or PKA/PKG, PP1 and/or 2A and tyrosine kinases is an important event for the 3F10-induced aggregation of thymocytes. The effects of metabolic inhibitors, which modulate the 3F10-induced aggregation of thymocytes observed semiquantitatively in microtitre plates, were also confirmed using a quantitative method (Table 1).

3F10 induces homotypic adhesion of thymocytes through an LFA-1-dependent/ICAM-1-independent pathway

Previous results suggest that signalling requirements for the 3F10-induced aggregation of thymocytes are similar with those mediated by $\beta 2$ integrins. To prove this hypothesis, we preincubated thymocytes with anti-CD11a (WT.1), anti-CD18 (WT.3) and anti-CD54 (1A29) mAb, and then stimulated with 3F10. Aggregation was measured quantitatively or semiquantitatively. As demonstrated in Fig. 6(a), the 3F10-induced aggregation was completely blocked by WT.3 and significantly inhibited by WT.1 mAb. In contrast, 1A29 mAb only slightly inhibited the aggregation. These results suggest that homotypic aggregation of thymocytes evoked by 3F10 is LFA-1 dependent/partly ICAM-1 dependent.

3F10 mAb potentiates thymocyte adhesion to the R-TNC.1 line

We have previously demonstrated that the R-TNC.1 cell line is

a type of rat cortical TEC with nursing activity which was manifested by the binding and subsequent engulfment of thymocytes.¹³ In this work, we studied the effect of 3F10 mAb on thymocyte binding to the R-TNC.1 line. As can be seen in Fig. 6(b) 3F10 also stimulated heterotypic adhesion of thymocytes to the R-TNC.1 line (stimulation was approximately 50%). Stimulatory effect of 3F10 mAb on thymocyte binding to R-TNC.1 cells was not simply a consequence of cross-linking these cells by mAb because preincubation of both thymocytes and the TEC line with saturation concentrations of 3F10 mAb also increased cell adhesion (data not shown). In order to determine whether this process is also mediated by $\beta 2$ integrins, we pretreated thymocytes with anti-LFA-1 or anti-CD18 mAb and R-TNC.1 cells with anti-ICAM-1 mAb for 30 min prior to the binding assay. Figure 6(b) shows that mAb to LFA-1 and CD18 partly suppressed thymocyte binding to R-TNC.1 cells, whereas anti-ICAM-1 mAb did not significantly modulate the adhesion process.

3F10 mAb stimulates proliferation of thymocytes triggered by Con A

Finally, we tested whether 3F10 modulates both spontaneous and Con A-induced proliferation of thymocytes. Results presented in Fig. 7 show that the mAb (at concentrations between 2.5 $\mu\text{g/ml}$ and 25 $\mu\text{g/ml}$) significantly stimulated proliferation of thymocytes triggered by the suboptimal Con A concentration (0.625 $\mu\text{g/ml}$). Maximal proliferation was seen using 5 $\mu\text{g/ml}$ of 3F10 mAb. 3F10 mAb did not significantly

Table 1. Requirements for 3F10- and PMA-induced aggregation of thymocytes

Chemical/condition	Aggregation		
	Semiquantitative		Quantitative 3F10 (%)
	3F10 (score)	PMA (score)	
Medium	2	3	39.99 ± 4.60
EDTA	0	0	NT
Mg ²⁺ Ca ²⁺ -free HBSS	0	0	NT
Mg ²⁺ (1 mM)	1	0	NT
Mg ²⁺ (2.5 mM)	2	1.5	NT
Mg ²⁺ (5 mM)	3.5	1	NT
Mg ²⁺ (1 mM) + Ca ²⁺ (5 mM)	3.5	2.5	NT
Mg ²⁺ (5 mM) + Ca ²⁺ (5 mM)	3.5	3.5	NT
Sodium azide (0.2%)	0	0	NT
Cytochalasin B (5 µg/ml)	0	0	NT
Cycloheximide (10 µg/ml)	2	3	NT
H-7 (80 µM)	1	3	25.63 ± 2.04*
HA 1004 (5 µM)	1	3	26.75 ± 7.30*
Genistein (50 µg/ml)	1	3	27.70 ± 8.70*
Okadaic acid (1 µM)	0	0	11.96 ± 2.58*
Bisindolylmaleimide (1 µM)	2	0.5	NT
Wortmannin (5 nM)	2	3	NT
W-7 (20 µM)	2	3	NT
Staurosporine (0.5 µM)	4	0.5	68.76 ± 1.85*

Thymocytes were cultured in the presence of 3F10 (25 µg/ml) and PMA (50 ng/ml). Where indicated, they were preincubated with inhibitors at 37° for 30 min before addition of 3F10 or PMA. Aggregation was scored after 2 h using semiquantitative aggregation assay. Effect of metabolic inhibitors which modulated the 3F10-induced aggregation of thymocytes observed semiquantitatively was also estimated using quantitative aggregation assay as described in Materials and Methods. Values are mean from four different experiments (semiquantitative method) or mean ± SD from three independent experiments (quantitative method). * *P* < 0.001 compared to the 3F10-induced aggregation. Spontaneous aggregation in medium alone was 9.80 ± 2.41. For studies of the divalent cation dependence, the cells were washed and resuspended in Mg²⁺ Ca²⁺ free HBSS medium and various concentrations of Mg²⁺ and Ca²⁺ were added as denoted in the table. NT = non tested.

modulate either spontaneous thymocyte proliferation or proliferation of these cells evoked by the optimal ConA concentration (2.5 µg/ml) (not shown).

DISCUSSION

The aim of the work presented in this paper was to explore the nature and function of the molecule detected by 3F10 mAb. This mAb was raised against rat cortical TEC line (R-TNC.1) previously established at the Institute of Medical Research, MMA, Belgrade.¹³ Immunohistochemistry and flow cytometry demonstrated that 3F10 mAb binds to both of thymocytes and thymic non-lymphoid cells, including the R-TNC.1 line. By these criteria, the 3F10 antigen belongs to a family of thymic shared molecules.^{2,3} Because the antigen is expressed on all TEC 3F10 mAb belongs to the group I of clusters of thymic epithelium staining (CTES) nomenclature.³

Immunoprecipitation experiments showed that 3F10 antigen is expressed in two forms with molecular masses of 65 and 55 000 MW. The two bands are identical in lysates from both

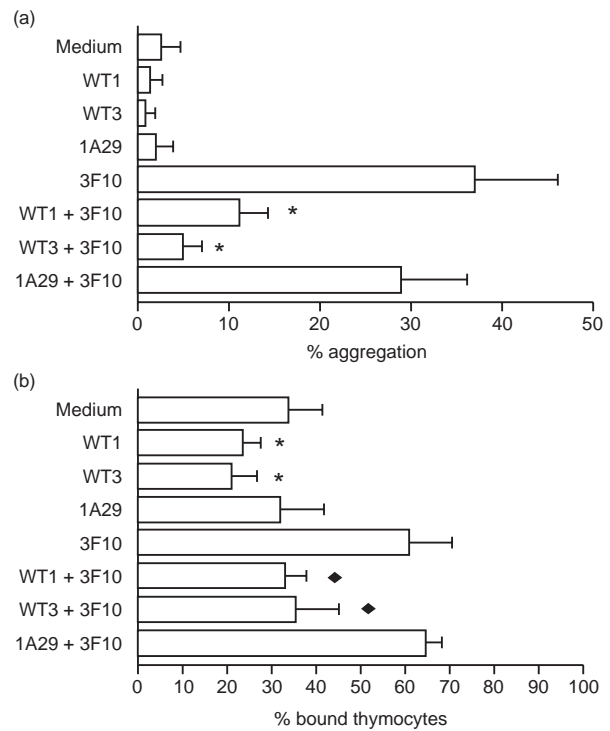


Figure 6. (a) Effect of WT1 (anti-CD11a), WT3 and 1A29 (anti-ICAM-1) mAb on homotypic aggregation of thymocytes triggered by 3F10. Cells were preincubated with these mAbs (10 µg/ml) for 30 min at 4° before addition of 3F10 mAb. Aggregation was measured quantitatively as described in Materials and Methods after 2 hr of incubation. Values are mean ± SD from three different experiments. **P* < 0.001 compared to the 3F10-induced thymocyte aggregation. (b) Effect of WT1 (anti-CD11a), WT3 (anti-CD18) and 1A29 (anti-ICAM-1) mAb on thymocyte binding to R-TNC.1 cells. Thymocyte binding was determined, as previously described after 45 min of cell culture at 37°. The antibody-mediated modulation of cell binding was tested by preincubating cells with mAb for 30 min at 4°. Values are mean ± SD from three different experiments. **P* < 0.01 compared to medium; ◆ *P* < 0.001 compared to 3F10.

thymocytes and the R-TNC.1 cell line. Because the molecules are detected by Western blotting, it can be postulated that each polypeptide carries the 3F10 epitope.

Based on cell distribution and biochemical characteristics of the 3F10 antigen, we proposed that the molecule might be the rat counterpart of mouse Crry/p65, a cell surface glycoprotein that belongs to the group of membrane-bound complement regulatory molecules.^{16,17} In humans, decay-accelerating factor (DAF, CD55), membrane cofactor protein (MCP, CD46), C8 binding protein (C8bp, homologous restriction factor of 60 000 MW, HRF), homologous restriction factor 20 (HRF20, CD59) and complement receptor 1 (CR1, CD35) have been reported as membrane-associated proteins which regulate complement activation.¹⁸⁻²⁰ One of the rat complement regulatory factors has been identified by 5I2 mAb.¹¹ Cloning experiments showed that the molecule possesses the same structure as mouse Crry/p65,¹⁴ which functions like DAF and/or MCP in humans.²¹ To confirm this hypothesis, we have compared the reactivity of 3F10 and 5I2 mAbs in Western blots of thymocyte lysates and with CHO

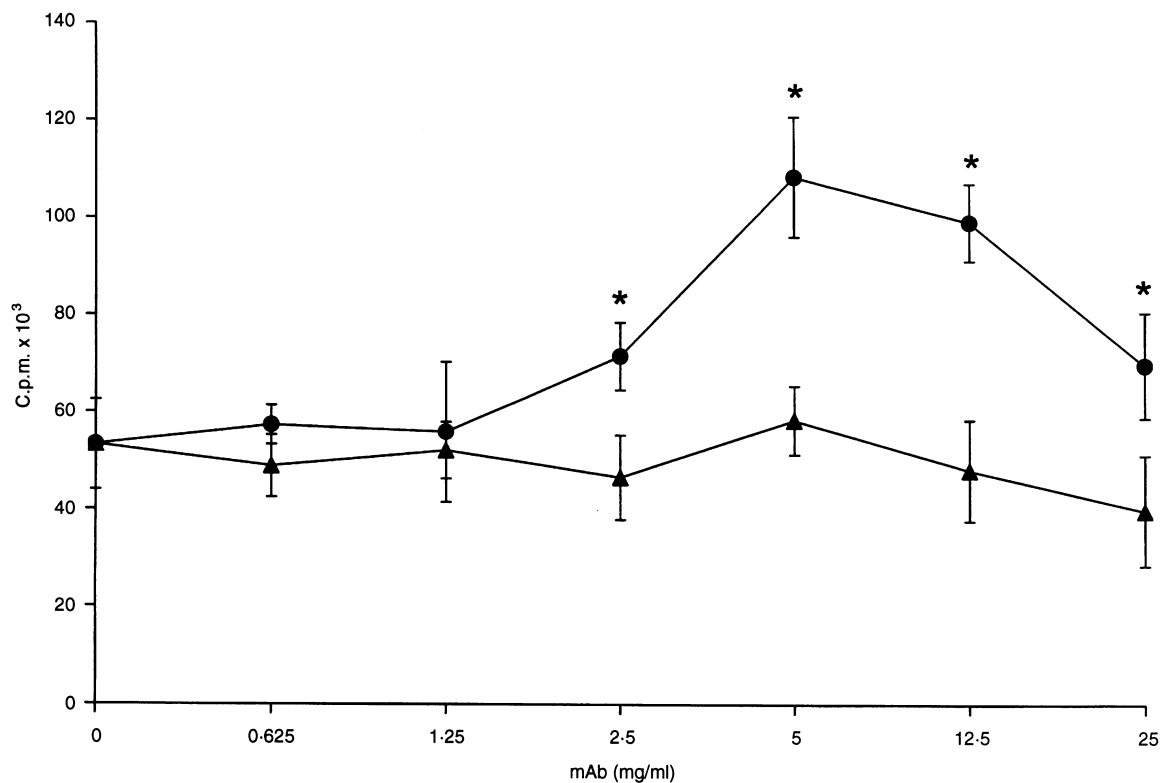


Figure 7. 3F10 mAb enhances proliferation of thymocytes stimulated by ConA. Thymocytes (1×10^6 /ml) were incubated with 0.625 μ g/ml Con A alone or with the addition of indicated concentrations of soluble 3F10 (circles) or IrmAb (triangles). The cells were cultivated for 3 days and [3 H]thymidine incorporation was measured as described in Materials and Methods. Values are given as mean \pm SD of triplicates of one representative experiment (out of three ones with similar results). * $P < 0.001$ compared to proliferation of thymocytes in the presence either Con A or IrmAb.

cells transfected with rat Crry by flow cytometry. We demonstrated that 3F10 and 5I2 mAb react with the identical molecules from thymocyte lysate and show the same reactivity with rat Crry transfected CHO cells. Therefore, the 3F10 antigen has been identified as the rat Crry/p65 molecule. However, these mAb show different epitope specificity (N. Okada, unpublished data).

We have shown that 3F10 mAb induces homotypic adhesion of rat thymocytes. 3F10-induced aggregation of these cells is dependent on the active cell metabolism, intact cytoskeleton and Mg^{2+} . Such dependence is characteristic for the integrin-mediated adhesion.²² The phenomenon was further confirmed by mAb blocking studies. We demonstrated that aggregation of thymocytes was completely blocked by anti-LFA-1 (anti-CD11a and anti-CD18) mAbs and was only partly inhibited by anti-ICAM-1 (CD54) mAb suggesting the involvement of LFA-1-dependent, ICAM-1-dependent and ICAM-1-independent adhesion pathways. Other ligands for LFA-1 in this model might be ICAM-2 or ICAM-3. However, the hypothesis has not been tested, because mAb to these molecules have not been available in the rat. We also have demonstrated that the engagement of Crry/p65 by 3F10 mAb stimulates heterotypic binding of thymocytes to cortical TEC line (R-TNC.1). The process was LFA-1 dependent but ICAM-1 independent. The aggregation process presented here is not specific only for 3F10 mAb, since engagement of a number of surface molecules on lymphocytes with ligands or mAb (CD2,

CD3-TCR, CD14, CD15, CD16, CD11a, CD18, CD19, surface immunoglobulin, CD39, CD40, CD43, CD44, MHC class II, CD99, CD81), generate proadhesive signals that activate LFA-1 molecules ('inside-out' signalling pathway), resulting in homotypic aggregation of lymphocytes.²³⁻²⁵ It has been suggested that these intracellular signals enhance the affinity or avidity of LFA-1 either by conformational changes or multimerization at the cell surface.²² Likewise, engagement of the 3F10 antigen can increase LFA-1-mediated adhesiveness through an 'inside-out' signalling pathway.

In further experiments, we tried to define potential intracellular signalling routes involved in thymocyte aggregation evoked by 3F10. To date, an exact understanding of the molecular steps that mediate activation of LFA-1 has remained elusive. In this context, PKC can serve as the second messenger for several of known stimuli. However, the use of different inhibitors suggests that more than one signalling pathways may play a role in activation of LFA-1 through the inside-out signalling and that both PK and PP activities have been implicated in the process.^{26,27} We showed that activation of PKC and/or A/G, PP1 and/or PP2A and tyrosine kinases is an important event for the 3F10-induced adhesion of thymocytes. Bisindolylmaleimide had no apparent effect, suggesting that 3F10 either did not activate PKC or signals generated upon binding of 3F10 mAb could act through PKC isoenzymes insensitive to this inhibitor. In contrast, adhesion evoked by PMA was suppressed by the inhibitor indicating that 3F10 and

PMA could use distinct activation mechanisms. The fact that 3F10 exerts an additive effect with PMA in inducing homotypic adhesion of thymocytes is in agreement with this assumption. Similarly, Pavlović *et al.*²⁸ have shown that NG2B12, a mAb directed to rat CD18, triggers an LFA-1 dependent thymocyte homotypic adhesion that utilizes distinct intracellular signalling routes in comparison to PMA. Similar results were reported by Petruzzelli *et al.*²⁹ using a model of homotypic aggregation of a B lymphoblastoid cell line (JY). They found that aggregation of JY cells stimulated by PMA is inhibited by staurosporine, whereas clustering of these cells induced by anti-CD18 mAb is staurosporine insensitive. In contrast, aggregation by both stimuli was completely blocked by pretreatment of the cells with inhibitors of protein tyrosine kinase (PTK) (genistein) and PP (okadaic acid). In our experiments, okadaic acid had inhibitory effect on both PMA- and 3F10-induced aggregation of thymocytes. Kansas *et al.*³⁰ have demonstrated that the LFA-1/ICAM-1-dependent homotypic adhesion of human B-cell lines induced by mAb reactive with CD19, CD20, CD39, CD40, CD43, and HLA-DR initiated signal transduction pathways that involves PTK, but not PKC. In contrast, Todd *et al.*²⁵ have shown that the anti-CD81-induced aggregation of thymocytes that is LFA-1 dependent, was inhibited by PKC, but not PTK inhibitors. All these reports, including results obtained in this study, suggest that activation of LFA-1 is mediated by different activation mechanisms depending on both engaged cell surface molecules and cell types.

We have also demonstrated that 3F10 mAb stimulates proliferation of thymocytes induced by suboptimal Con A concentrations, indicating that the rat Crry/p65 is involved in activation of thymocytes. The described functions of the antigen have not been published either for the mouse Crry/p65 or for its functional homologues in humans.

In conclusion, in the present work we demonstrated that 3F10 mAb, specific for the rat homologue of mouse Crry/p65, stimulate homotypic adhesion and activation of thymocytes and binding of thymocytes to TEC. These results suggest that 3F10-derived signals may regulate the thymocyte/TEC adhesiveness through activation of LFA-1. In the context of thymocyte development, the regulation of cell adhesiveness is of crucial importance. Up to now, a large number of receptor/ligand interactions have been described in the thymus.⁴ In addition, many different signals lead to the activation of LFA-1.^{23–25} We demonstrated for the first time that engagement of the Crry/p65 molecule enhances cell adhesiveness and activation of thymocytes through LFA-1 and that the phenomenon may be relevant for selection processes of T cells in the thymus.

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