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Expression of Immunoglobulin Superfamily Cell Adhesion Molecules on Murine Embryonic Stem Cells[1]

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ABSTRACT

The expression of cell adhesion molecules of the Ig superfamily (Ig-CAM) were examined on embryonic stem (ES) cells during culture in vitro. ES cells maintained an undifferentiated phenotype when cultured in the presence of leukemia inhibitory factor (LIF) or with fibroblast feeder cells; > 90% of cells reacted positively to an antibody (ECMA-7) that marks undifferentiated ES cells. Using flow cytometry, high concentrations of ICAM-1, VCAM-1, and NCAM antigens were detected on undifferentiated ES cells, but their specific receptors, Mac-1, LFA-1, and VLA-4, were not detected. There was also no class I or II major histocompatibility complex (MHC) antigen expression. The ICAM-1 expressed was functional, since anti-ICAM-1 significantly ($p < 0.0001$) blocked ES cell-lymphocyte binding. Ig-CAM and MHC-1 expression on undifferentiated ES cells was not up-regulated by treatment of cells with interferon-γ (IFN-γ), tumor necrosis factor α, or flavivirus infection, agents that up-regulate these molecules in other embryonic cell types. Twelve hours after LIF withdrawal, ICAM-1 and NCAM expression decreased significantly, while VCAM-1 was undetectable. However, morphology and ECMA-7 expression remained unchanged. Similar patterns of expression were seen on ES cells maintained on fibroblast feeder cells. This suggests that LIF or other cytokines may maintain the expression of Ig-CAMs on undifferentiated cells. Differentiation was induced by dimethyl sulfoxide treatment for 14 days. Cells changed from a colony-forming to a monolayer morphology, and ~60% of the cell population no longer expressed ECMA-7. In these cells, VCAM-1 was undetectable and ICAM-1 and NCAM had declined to low levels. In these differentiated cells, ICAM-1 and MHC-1 were inducible by IFN-γ. This study suggests that the pattern of expression of the Ig-CAMs in ES cells may have a role in defining the phenotype of differentiated and undifferentiated cells.

INTRODUCTION

Embryonic stem (ES) cells are pluripotent cells that are similar in phenotype to inner cell mass cells of embryos. They can be induced to undergo differentiation in vitro that closely resembles differentiation in the normal embryo [1]. ES cells can be established in primary culture and have proved useful for the study of some aspects of early embryo development and differentiation.

ES cells can be cultured in vitro for 30–50 cell generations. The presence of leukemia inhibitory factor (LIF) [2] or culture on somatic cell feeder layers [3] maintains the undifferentiated state of the cells. ES cells can be induced to differentiate along various pathways, depending on culture conditions, readily committing to mesenchymal [1] and lympho-hematopoietic [4–6] as well as other lineages. A common feature of ES cells following induction of differentiation is a change of cell phenotype from colony-forming to monolayer morphology. This change in the nature of cell-cell and cell-substratum interactions may suggest that the status of intercellular adhesion molecules on these cells may change upon differentiation.

Four adhesion molecule families, i.e., the immunoglobulin (Ig) superfamily, the integrins, homing cellular adhesion molecules, and the cadherins, have been described to date. In the present report, the first of a series of reports on investigations into the status of these adhesion molecules on ES cells, we examine the Ig superfamily of cell adhesion molecules (Ig-CAMs). The Ig superfamily includes molecules with an immunoglobulin structure such as ICAM-1, ICAM-3, VCAM-1, NCAM, class I and II major histocompatibility complex (MHC) molecules, LFA-2, LFA-3, TCR/CD3, CD4, and CD8. Except for NCAM, these molecules were originally studied in the adult immune system. Recently some of these, including ICAM-1 and VCAM-1, have been found to be expressed on the early human preimplantation embryo [7]. Furthermore, ICAM-1 expression can be up-regulated on midterm murine trophoblast cells by cytokine treatment, which increases the sensitivity of the cells to lysis by paternal-specific cytotoxic T cells [8].

This report shows that ICAM-1, NCAM, and VCAM-1 (but not MHC-I) antigens were expressed at high levels on undifferentiated ES cells, but down-regulated soon after the induction of differentiation. Expression in undifferentiated cells may be constitutive, and it was not influenced by cytokines or virus infection. In contrast, ICAM-1 expression in differentiated cells was up-regulated by interferon-γ (IFN-γ). These differences in patterns of expression suggest that Ig-CAM molecules may be part of the overall phenotype of differentiated and undifferentiated ES cells.

MATERIALS AND METHODS

Murine Embryonic Cell Lines and Cell Culture

ES cell lines were a generous gift from Dr. L. Williams (School of Veterinary Science, the University of Melbourne, Australia). The lines were received at passage 8, D3 (strain: 129/Sv+/+, XY karyotype) and passage 14, MBL-5 (strain: 129/Sv He, XY karyotype) [9]. Cell lines were cultured in Dulbecco's Modified Eagle's medium (DMEM) (MultiCel; Cytosystems Pty. Ltd., Castle Hill, NSW, Australia) supplemented with murine recombinant LIF (AMRAD Corporation Ltd., Boronia, Victoria, Australia) (1000 U/ml [10]), 10% fetal calf serum (FCS), 0.1 mM mercaptoethanol, 200 mg/ml streptomycin, and 200 U/ml penicillin G under standard culture conditions (SCC), i.e., 5% CO_2 and humidified air at 37°C. ES were cultured in 25-cm^2 flasks (Corning 25100; Dow Corning, Corning, NY) at a density of $2 \times 10^4/cm^2$ and subcultured every 3 days to maintain pluripotency. At subculture, cells were

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washed first with Dulbecco's PBS (Sigma Chemical Co., St. Louis, MO); then Hanks' Ca^{2+}/Mg^{2+}-free buffered saline supplemented with 0.1% trypsin and 1 mM EDTA (Sigma) was added for 2 min. Trypsinization was stopped by adding DMEM containing 10% FCS. Cells used in the experiments were between 12 and 21 passages. ES cells of the D3 line were injected into blastocysts at passage 21, and normal chimeras were subsequently born. Unless mentioned, ES cells of the D3 line were used in this study.

ES cells were also maintained in an undifferentiated state by culture on feeder layers. Primary mouse embryonic fibroblasts (MEF) isolated from Day 14 BALB/c embryos were used as feeder cells. Isolation of MEF has been described in detail elsewhere [11]. Briefly, feeder cells were irradiated with 3000 rad (30 Gy) and then seeded in 25-cm^2 plastic tissue culture flasks (Corning 25100) overnight at a density of 2.5×10^5 per flask in 5 ml of DMEM. ES cells were then seeded onto the feeder layer at a density of $2 \times 10^4/cm^2$. ES cells were subcultured every 3 days to maintain pluripotency. By this time the feeder cells had begun to lyse. Subculture procedures used were as described above. ES cells cultured on feeder layers were subjected to flow cytometric analysis in order to compare the CAM expression with that seen in the presence of LIF. Since there was no apparent difference in the CAM expression or culture behavior between cells cultured on fibroblast feeder layers and those cultured in the presence of LIF, and since LIF is a single, well-defined reagent, the data from experiments making use of LIF are presented here.

Cytokine Treatment

ES cells were exposed to IFN-γ and tumor necrosis factor α (TNFα) at concentrations ranging from 300 U to 3000 U/ml for 24, 48, and 72 h. In undifferentiated ES cells, none of these treatment protocols altered the CAM expression in ES cells. Therefore we used 500 U/ml for IFN-γ [8] and 1000 U/ml for TNFα and an incubation period of 48 h, as these concentrations and the incubation period induce maximal expression of ICAM-1 and MHC-I molecules in MEF (L. Tian and N.J.C. King, unpublished data). Recombinant murine IFN-γ and TNFα were kind gifts of Boehringer Ingelheim, Sydney, Australia.

Induction of ES Cell Differentiation In Vitro

The induction method used has been described previously [1]. Briefly, to induce differentiation, ES were trypsinized with 0.1% trypsin and 1 mM EDTA and plated at a density of 2×10^5/ml in bacterial grade (nonadherent surface) petri dishes, in DMEM without LIF, for 48 h. Cell aggregates were collected and cultured in tissue culture flasks in DMEM containing 0.1% dimethyl sulfoxide (DMSO) (Ajax Chemicals; Crown Scientific, Moorebank, NSW, Australia), and the medium containing DMSO was replaced every 2 days. Morphologically mixed cell populations were seen at Day 14, with ES cells and neural-, muscle-, and fibroblast-like cells in various proportions. Differentiated cells were removed with trypsin-EDTA for labeling and flow cytometry.

Virus Stocks and Virus Infection

West Nile virus (WNV) was seeded at a multiplicity of infection (m.o.i.) of 5 onto vero cells grown in DMEM supplemented with FCS and antibiotics, as described above under SCC, and harvested after 30 h, as previously described [12]. Virus titers were determined by serial dilutions on vero cell monolayers as described previously [13]. Virus stocks were frozen at −80°C and thawed immediately prior to use.

ES cells for flow cytometric assay were seeded in 6-well plates (Nunclon; Nunc, Roskilde, Denmark) at a density of 2×10^4 cell/cm^2 for 16 h. They were infected with WNV at 5–20 plaque-forming units (pfu) per cell for 1 h or were mock-infected. The virus was then aspirated, 2 ml of culture medium was added into each well, and the cells were incubated for a further 24 or 48 h before cell labeling and flow cytometry. This range of virus concentrations has previously been used to induce maximal ICAM-1 and MHC expression [14].

Infection of ES cells was confirmed in parallel by immunofluorescence microscopy. ES cells seeded onto sterile gelatine-coated coverslips at a density of 2×10^4 cell/cm^2 for 16 h were infected or mock-infected as described above. On the assay day, cells on coverslips were fixed in acetone for 20 min at 4°C and labeled with polyclonal anti-WNV antibody or nonspecific control (preimmune) antibody for 1 h. Cells were washed and labeled with secondary fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG (#DAF, Silenus, North Ryde, NSW, Australia) for 1 h at 4°C. Washed cells were mounted on slides in DABCO (1,4 diazabicyclo-[2.2.2]octane; Sigma) anti-fade glycerol [15]. Photographs were taken using a Leica fluorescence microscope connected to a Photoautomate (Leica, North Ryde, NSW, Australia). Kodak Ektachrome P800/1600 film (Eastman Kodak, Rochester, NY) was used.

Monoclonal Antibodies

The CD designations of the studied cell adhesion molecules are as follows: CD54—ICAM-1; CD102—VCAM-1; CD56—NCAM; CD49d/CD29—VLA-4; CD11a/CD18—LFA-1; and CD11b/CD18—Mac-1. Primary monoclonal antibodies against surface antigens were used as follows: anti-ICAM-1 (hybridoma supernatant and purified antibody, clone: YN1/1.7.4, ATCC# CRL 1878 [American Type Culture Collection [Rockville, MD]; also purified antibody, clone: 3E2, Pharmingen, Bioclone, Marrackville, NSW, Australia), anti-VCAM-1 (purified antibody, clone: MVCAM.A 492, Pharmingen USA, San Diego, CA), anti-NCAM (purified antibody, clone: H28.123, Immunotech, Coulter, Adelaide, SA, Australia), anti-H-$2K^bD^b$ (hybridoma supernatant, ATCC# HB-11), anti-I-A^b (hybridoma supernatant, ATCC# HB-38), anti-LFA-1 (purified antibody, clone: 2D7, Pharmingen USA; hybridoma supernatant, ATCC# TIB-217 M17/4.4, anti-α subunit), anti-Mac-1, anti-α subunit (hybridoma supernatant, ATCC# TIB-128), anti-VLA-4, α subunit, (purified antibody, clone: 428, Seikagaku Corporation, Tokyo, Japan). ECMA-7 antibody (kind gift of Dr. L. Williams) was used as a marker [10, 16] to identify ES cells. For isotype control labeling, the following antibodies were used: anti-mouse IgG_{2a}, IgG_{2b} (purified; Silenus), and IgM (purified antibody; Serotec; Australian Laboratory Services, Rockdale, NSW Australia). The following secondary FITC-conjugated antibodies were used: anti-rat IgG (Silenus) and anti-mouse IgM (Serotec).

In order to quantitate cell-surface molecules, all antibodies were carefully titrated on relevant high-expressing cells to ensure saturation labeling on ES cells.

Sample Preparation and Cell Labeling for Flow Cytometry

On the assay day, ES cells were removed from flasks using 0.1% trypsin plus 1 mM EDTA in PBS at 37°C for 2 min. This treatment does not alter the serological detectability of MHC or ICAM-1 molecules [17, 18]. To confirm this in ES cells, ES cells were removed with a silicon cell scraper when they were seeded sparsely (data not shown). Then 2×10^5 cells per sample were transferred into labeling tubes and incubated with primary antibodies diluted appropriately in 100 μl of culture medium for 60 min at 4°C. After incubation, the cells were centrifuged through 100 μl FCS; the supernatant containing unbound antibody was aspirated, and this was followed by secondary FITC-conjugated antibody labeling for 60 min at 4°C. After this incubation, cells were spun through an FCS bed as described above and resuspended in 400 μl PBS and kept at 4°C for flow cytometry. At this time > 95% of cells were live as evidenced by trypan blue exclusion.

Flow Cytometry

Fluorescence between 515 and 545 nm (FITC) or between 565 and 605 nm (propidium iodide) was measured using a FACScan (Becton Dickinson, Rutherford, NJ) equipped with an argon ion laser set at 488 nm. Forward and side scatter measurements were within the same range for all populations, indicating that there were no cell size changes arising from the different treatment protocols used. Only live cells were gated for FITC fluorescence (i.e., cell-surface molecule labeling) analysis; any cells incorporating propidium iodide into the nucleus were excluded from the assay as being dead. Since possible capping and antibody endocytosis was inhibited by the use of low temperature incubations, it follows that only antigen expressed on the cell surface and labeled by the relevant antibody there was analyzed in these assays. The FACScan setup was electronically identical for each experiment, and 10 000 gated events were counted for each sample. Data analysis was performed using the Lysis II program (Becton Dickinson, Mountain View, CA), and the results are presented either as histograms or as bar graphs. Data illustrating representative results from individual experiments (in Figs. 1, 2, 6a, and 7) are presented in histograms as cell number versus $\log_{10}$ fluorescence intensity. Percentage positive cells was taken as the percentage of the cell population exhibiting a fluorescence greater than that of the top 10% of fluorescence of the comparative (antibody isotype or untreated) control cells. For representation as bar graphs (in Figs. 3 and 6b), mean fluorescence was calculated and used as a measure of relative CAM expression. Data were normalized against the isotype control fluorescence and amalgamated from at least three experiments. Each value shown represents mean population fluorescence averaged over all experiments (± SD) as an indication of cell-surface CAM expression.

ES-Lymphocyte Binding, ^{51}Cr Release Assay

Activated syngeneic (C57/BL6, H-2^b) splenocytes harvested from spleens of C57/BL6 mice were used for leukocyte-ES cell binding assays as described previously [19]. Briefly, splenocytes were centrifuged through a density bed made up of Ficoll 400 (Pharmacia Biotech AB, Uppsala, Sweden), 14% (w:v) in water, and 32.8% sodium metrizoate (Sigma) to separate the red cells and dead cells. Live leukocytes taken from the density interface were activated by stimulating with phorbol myristate acetate (Sigma) at a concentration of 10 ng/ml for 4 h. Activated leukocytes were labeled with ^{51}Cr ($Na_2{}^{51}CrO_4$ NEZ-030S; Dupont, North Sydney, NSW, Australia) for 1 h in a polypropylene centrifuge tube at a concentration of 300 μCi/2×10^7 cells. In the blocking assay, purified anti-ICAM-1 (hybridoma YN1/1.7.4) antibody was used at a final concentration of 30 μg/ml in the culture medium. This concentration was optimal and was determined by titration of the antibody in ES cells. Purified IgG2a (final concentration, 30 μg/ml; Silenus) was used as a negative isotype antibody control. ES cells were seeded in 96-well plates at 2×10^4 cells per well for 16 h in replicates of 6. Cells were incubated with anti-ICAM-1 antibody, with IgG2a isotype control antibody, or without antibody for 1 h at 4°C in 100 μl of culture medium. After incubation they were washed twice in PBS, and 2×10^5 radiolabeled leukocytes were then added into each well for the binding assay. ES cells were incubated with activated radiolabeled leukocytes for a further 3 h under SCC. To terminate the assay, the leukocyte-containing medium was discarded and the ES cells were washed vigorously three times with PBS. After this, 200 μl of 0.25% Triton X-100 was added to each well to lyse bound leukocytes. Then 100 μl of the resulting supernatant was taken and mixed with 3 ml of scintillant cocktail (Emulsifier Safe; Packard, Sydney, Australia). The radioactivity was measured in a Beckman LS6000 (Palo Alto, CA) liquid scintillation counter as counts per minute.

Statistical Analysis

For statistical comparisons of only two groups, an unpaired, two-tailed Student's *t*-test was used. A p value of < 0.05 was considered significant. For simultaneous analysis of three or more groups, a one-way ANOVA was employed with the posttest utilizing the Tukey-Kramer multiple comparisons test. InStat 2.03 from GraphPad (San Diego, CA) software was used in this statistical analysis.

RESULTS

Identification of ES Cells

The identification of ES cells was made using ECMA-7 antibody, which was shown to have specificity for pluripotent ES cells [10, 16]. All undifferentiated ES cells used in the experiments were 90–94% ECMA-7 positive. Figure 1a shows that 92.8% of ES cells were labeled by ECMA-7 as compared to IgM isotype background control (passage 14).

Expression ICAM-1, VCAM-1, NCAM, and MHC Antigens on ES Cells

Two anti-ICAM-1 monoclonal antibodies obtained from different clones (YN1/1.7.4 and 3E2) were used on both ES cell lines, D3 and MBL-5. Flow cytometry using either antibody consistently showed that ES cells expressed high levels of ICAM-1, 15- to 20-fold higher than isotype background fluorescence on both cell lines. Figure 1b shows that ICAM-1 (profile D) was detected at a 17.5-fold higher level than background isotype labeling (profile A).

VCAM-1 was expressed in the same sample at about 9-fold higher levels than background labeling (Fig. 1b, profile C), while NCAM was detected at approximately 7-fold higher levels than background labeling (Fig. 1b, profile B). These results were consistent for all passages tested.

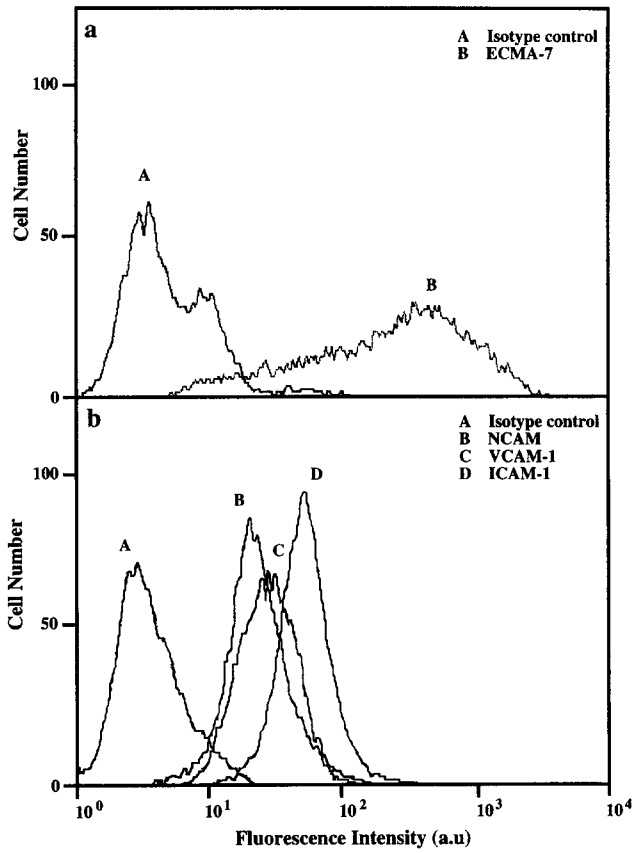

FIG. 1. **a**) Representative overlaid flow cytometric histograms of passage 14 ES cells labeled with the cell-surface ES cell marker antibody, ECMA-7. The data show that 92.8% of ES cells were labeled with ECMA-7 antibody as compared to the IgM isotype control. The percentage positive cells was taken as the percentage of the cell population exhibiting a fluorescence greater than that of the top 10% of fluorescence of the isotype control cells. **b**) Representative overlaid flow cytometric histograms of passage 11 ES cells labeled with antibodies to the cell-surface adhesion molecule markers, ICAM-1, VCAM-1, and NCAM. Comparison to the isotype control showed that the relative population fluorescence intensities were 17.5-fold, 9-fold, and 7-fold that of the isotype control, respectively. Fluorescence instensity is measured in arbitrary units.

MHC class I and II (MHC-I and MHC-II) antigens were not detected above the background levels of the isotype control antibody on ES cells at any passage number.

Expression of ICAM-1 and VCAM-1 Receptors

In view of the high expression of ICAM-1 and VCAM-1, we determined whether either LFA-1 or Mac-1 (defined receptors in immune responses for ICAM-1) or VLA-4 (a receptor for VCAM-1) were expressed on these cells. Monoclonal antibodies (each reacting only with the respective identifying α subunit) failed to detect surface LFA-1, Mac-1, or VLA-4 (Fig. 2, b, c, and e), as compared to the respective isotype controls (Fig. 2, a and d).

Inducibility of CAM on ES Cells

ES cells were exposed to 100 U/ml-3000 U/ml IFN-γ and TNFα for 48 h. Flow cytometry showed that no up-regulation of ICAM-1, VCAM-1, or NCAM occurred on ES cells at any concentration for either cytokine. Figure 3a shows the mean fluorescence intensity (± SD) from three experiments. MHC-I, MHC-II, LFA-1, Mac-1, and VLA-4 expression also remained undetectable after treatment with these cytokines (data not shown).

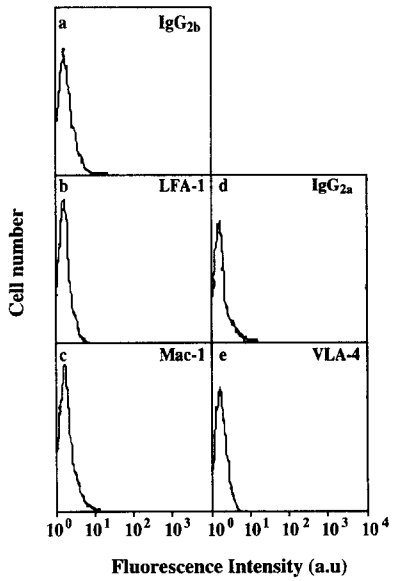

FIG. 2. Representative flow cytometric histograms of passage 11 ES cells labeled with antibodies to the identifying α subunit of cell-surface integrins, LFA-1 (**b**), Mac-1 (**c**) (ICAM-1 receptors), and VLA-4 (**e**) (VCAM-1 receptor). Compared to the respective isotype controls (**a, d**), there was no detectable expression of LFA-1 or Mac-1, or of VLA-4. Fluorescence intensity is measured in arbitrary units.

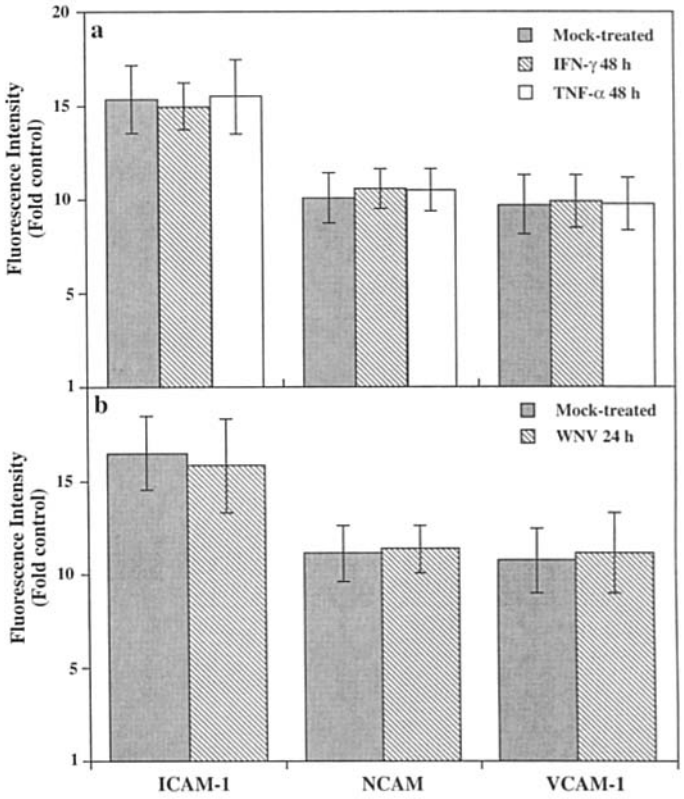

FIG. 3. **a**) Expression of ICAM-1, NCAM, and VCAM-1 on passage 14 ES cells after cytokine treatment (IFN-γ, 500 U/ml; TNFα, 1000 U/ml). The data show no detectable change in the expression of these CAMs after these treatments. **b**) Expression of ICAM-1, NCAM, and VCAM-1 on passage 14 ES cells after WNV infection at 20 pfu per cell. The data show no detectable change in the expression of these CAMs after WNV infection. In both cases, data were normalized against the isotype control fluorescence and amalgamated from at least three experiments. Each value shown represents mean population fluorescence averaged over all experiments (± SD) as an indication of cell-surface CAM expression.

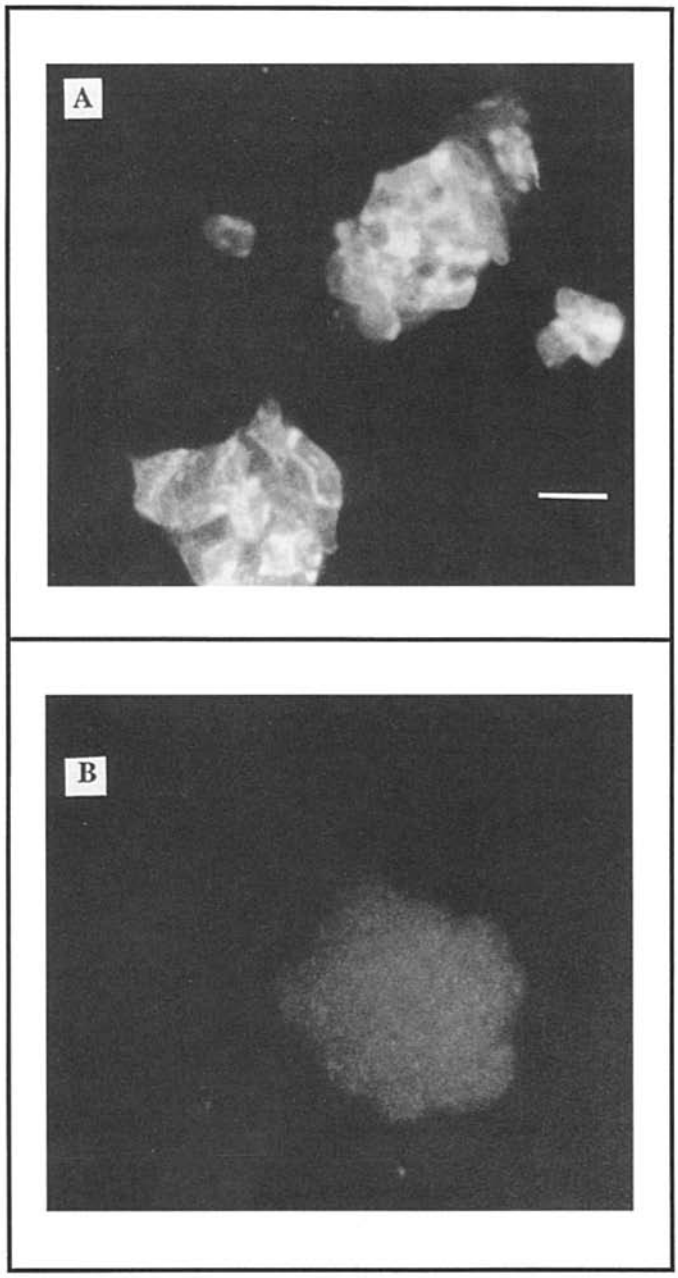

FIG. 4. Infection of ES cells by WNV. **A**) ES cell colonies infected with WNV, 20 pfu per cell for 24 h, and then labeled with a primary anti-WNV antibody and secondary FITC-conjugated antibody. In infected cultures, brightly labeled cells can be seen in WNV-infected colonies as compared to mock-infected ES cell colonies (**B**), labeled identically to and at the same magnification as those in **A**. Bar = 3.9 μm.

WNV, a member of the flavivirus family of arboviruses, can directly up-regulate ICAM-1 and MHC-I and -II [14, 17, 20] as well as VCAM-1 (unpublished data) in human embryonic fibroblasts. ES cells were infected with WNV at m.o.i. ranging from 5 to 20 pfu per cell. Infection times were kept to 24 h to maintain single-step kinetics. The expression of ICAM-1, VCAM-1, and NCAM was not altered after infection at any of these m.o.i. (Fig. 3b); nor did LFA-1, Mac-1, VLA-4, MHC-I, or MHC-II expression become detectable.

Infection was confirmed by using primary polyclonal mouse anti-WNV antibody and FITC-conjugated secondary antibody to label infected, acetone-fixed ES cells on coverslips and by comparing these to both uninfected and infected ES cells labeled with preimmune mouse serum. This is shown in Figure 4 for ES cells infected at 20 pfu per cell. WNV-infected ES cells labeled with anti-WNV antibody (panel A) showed greater fluorescence throughout the cytoplasm than similarly labeled, uninfected cells (panel B) or infected cells incubated with preimmune mouse serum (not shown). Between 30% (if ES cells were infected at m.o.i. of 5 pfu per cell) and 70% (if cells were infected at m.o.i. of 20 pfu per cell) of ES cells in culture were detectably infected. In all cases, irrespective of the m.o.i. used to infect the cells, greater than 95% of cells were viable as determined by trypan blue exclusion.

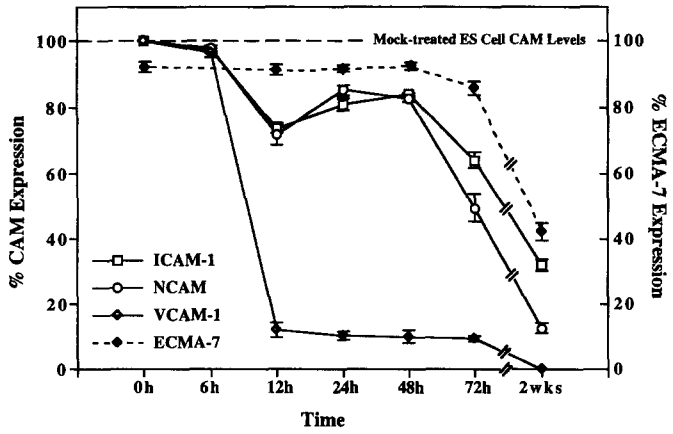

FIG. 5. The kinetics of change in ICAM-1, NCAM, and VCAM-1 expression on passage 14 ES cells after withdrawal of LIF for varying times. The data show no reduction in CAM expression at 6 h after LIF withdrawal. Significant reduction in ICAM-1 ($p = 0.001$) and NCAM ($p = 0.001$) expression occurred by 12 h, with a dramatic and sustained reduction in VCAM-1 expression ($p < 0.0001$). No significant change in ECMA-7 labeling was seen in this 72-h period. The 2-wk time point for DMSO treatment in this population is also shown for comparison. For % CAM expression values, mean population fluorescence data were normalized against the mean isotype control fluorescence in each experiment. This value was then expressed as a percentage of the mock-treated control mean population fluorescence (shown as the 100% line in the figure). Each value represents this percentage averaged over three experiments (± SD). Percentage ECMA-7 expression values represent the percentage of the cell population expressing this marker. The percentage positive cells was taken as the percentage of the cell population exhibiting a fluorescence greater than that of the top 10% of fluorescence of the isotype control cells. Each value represents this percentage averaged over three experiments (± SD).

Functional Confirmation of ICAM-1 Expression on ES Cells

A lymphocyte-ES cell adhesion assay was used to test whether the serologically detected ICAM-1 was functional. Phorbol myristate acetate-activated splenocytes (C57BL/6, H-2^b, i.e., syngeneic cells) labeled with ^{51}Cr were used in binding assays with the D3 ES line. Six replicates were performed. From a representative experiment, the mean values (± SD) for maximal control binding (without antibody), binding in the presence of a polyclonal isotype control antibody (IgG2a), and binding in the presence of anti-ICAM-1 antibody were 14424 ± 1052, 11210 ± 475, and 7223 ± 359 counts per minute, respectively. From this data it can be seen that the binding between ES and lymphocytes was significantly blocked by anti-ICAM-1 antibody. Although some inhibition of lymphocyte-ES cell adhesion occurred in cultures incubated with nonspecific polyclonal isotype antibody, as compared with cultures incubated without antibody, nevertheless 35.6% blocking occurred in anti-

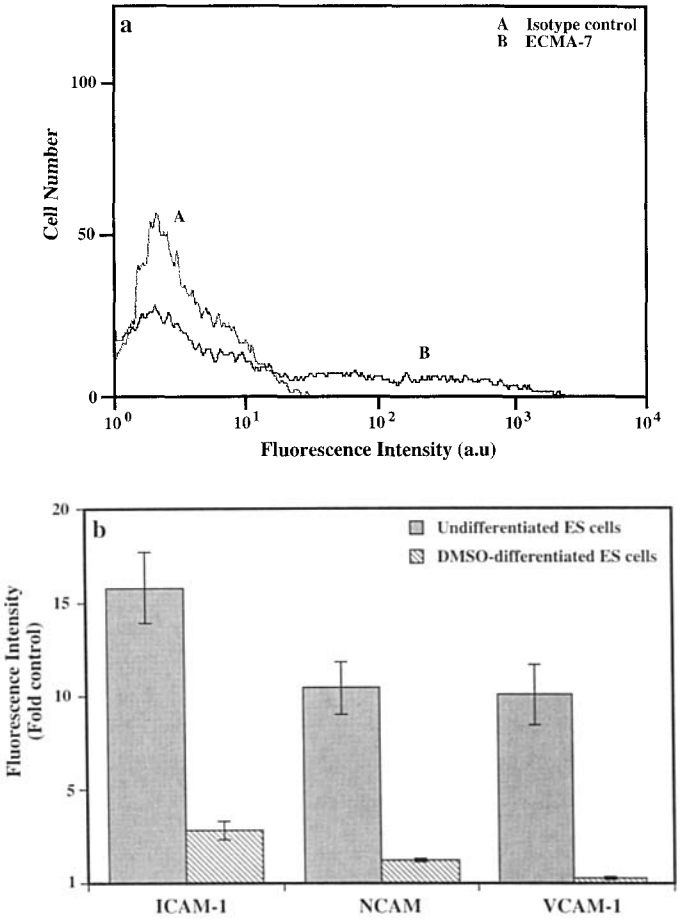

FIG. 6. **a**) Representative overlaid flow cytometric histograms of 2-wk DMSO-differentiated, passage 14 ES cells labeled with the cell-surface ES cell marker antibody, ECMA-7. The data show that 42% of ES cells were labeled with ECMA-7 antibody as compared to the IgM isotype control. Fluorescence intensity is measured in arbitrary units. The percentage positive cells was taken as the percentage of the cell population exhibiting a fluorescence greater than that of the top 10% of fluorescence of the isotype control cells. **b**) Expression of ICAM-1, NCAM, and VCAM-1 on passage 14 ES cells differentiated with DMSO for 2 wk. The data show a significant reduction in the expression of ICAM-1 ($p = 0.003$) and NCAM ($p = 0.0037$), with disappearance of detectable VCAM-1 ($p < 0.0005$) after DMSO differentiation. Data were normalized against the isotype control fluorescence and amalgamated from at least three experiments. Each value shown represents mean population fluorescence averaged over all experiments (± SD) as an indication of cell-surface CAM expression.

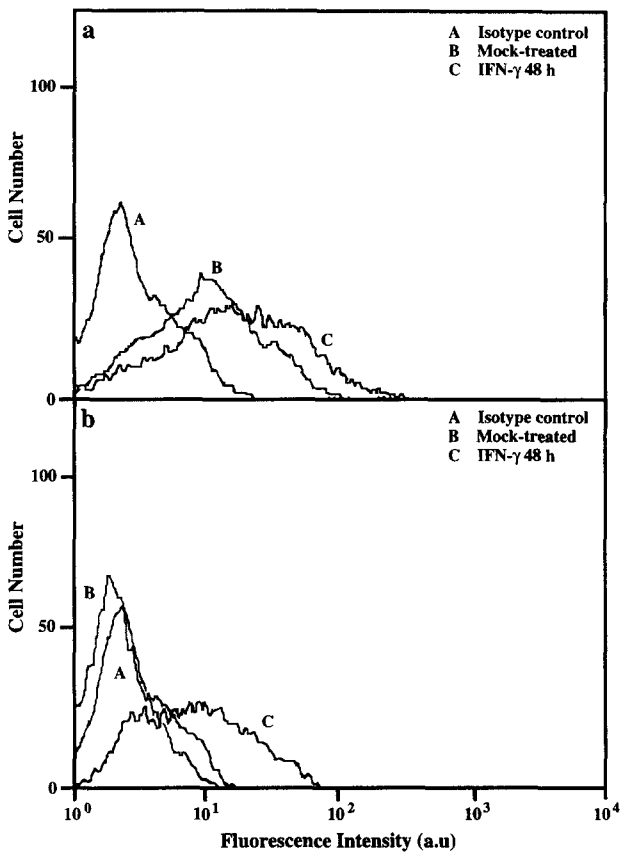

FIG. 7. **a**) Representative overlaid flow cytometric histograms of 2-wk DMSO-differentiated, passage 14 ES cells after 48 h IFN-γ treatment or mock treatment, and labeling for ICAM-1. Compared to the value for the isotype control, the relative population fluorescence intensities were 10-fold and 5-fold, respectively. This indicates constitutive ICAM-1 expression by these cells and a readily detectable response to IFN-γ. **b**) Representative overlaid flow cytometric histograms of 2-wk DMSO-differentiated, passage 14 ES cells after 48 h IFN-γ treatment or mock treatment, and labeling for MHC-I. Compared to the isotype control, there was no detectable constitutive MHC-I on mock-treated DMSO-differentiated ES cells. However, the relative population fluorescence intensity increased by some 5-fold after 48 h IFN-γ treatment. Fluorescence is measured in arbitrary units.

ICAM-1 antibody-incubated cultures as compared to those incubated with nonspecific polyclonal isotype antibody. This level increases to 49.9% blocking when compared to cultures incubated without antibody. Thus ICAM-1 expressed by the ES cells was recognized by activated leukocytes, confirming the functional integrity of the molecule.

CAM Expression in Differentiating ES Cells

The effect of LIF withdrawal was tested. LIF was removed from ES cell cultures; thus ES cells were cultured in DMEM without LIF, and the expression of ICAM-1, VCAM-1, NCAM, and ECMA-7 was determined at 6, 12, 24, 48, and 72 h after withdrawal (Fig. 5). Cell morphology was also assessed at these times by phase-contrast microscopy. The data were analyzed using a one-way ANOVA and the Tukey-Kramer multiple comparisons posttest. No significant ($p > 0.05$) change in CAM expression occurred after 6 h. By 12 h, VCAM-1 expression was almost completely lost ($p < 0.0001$), and this persisted for the 72 h of culture. A significant reduction in ICAM-1 ($p < 0.001$) and NCAM ($p < 0.001$) expression occurred at 12 h after LIF was withdrawn, and these values decreased further (to about 50% of vehicle-treated levels) by 72 h. No significant change ($p > 0.05$) in ECMA-7 labeling, or change in ES cell morphology, was seen for the 72 h after LIF withdrawal.

Differentiation was induced by culturing cell aggregates in DMEM plus 10% FCS and 0.1% DMSO for 2 wk without LIF and without subculturing [1]. Using this technique, morphologically mixed cell populations were obtained that were consistent with differentiation to neural-, muscle-, and fibroblast-like lineages. The cells were removed from the plastic flasks by 0.1% trypsin and 1 mM EDTA for labeling and flow cytometry. Flow cytometric results showed that compared to isotype control labeling, ECMA-7 labeling was reduced to 42% positive in the DMSO-treated cells (Fig. 6a).

Cell-surface expression of ICAM-1 ($p = 0.01$), VCAM-1 ($p < 0.01$), and NCAM ($p < 0.001$) was also reduced on ES cells after 14 days of DMSO treatment (Fig. 6b). While the expression of ICAM-1 and NCAM was

TABLE 1. Summary of adhesion molecules detected by monoclonal antibodies and flow cytometry in ES cells.

Adhesion molecules (mAb clone)	Early passage ES cells			Differentiated ES cells		
	Untreated	IFN-γ-treated	WNV-infected	Untreated	IFN-γ-treated	WNV-infected
ICAM-1 (YN-1/1.7.4)	+++	+++	+++	+	++	+
NCAM (H28.123)	+++	+++	+++	+	+	+
VCAM-1 (MVCAM.A)	+++	+++	+++	−	−	−

markedly reduced, the expression of VCAM-1 became undetectable compared to background control. Induction of differentiation with DMSO had no effect on the expression of MHC-I, MHC-II, LFA-1, Mac-1, or VLA-4 antigens, all of which remained undetectable (data not shown).

Induction of ICAM-1 and MHC-I by IFN-γ Treatment in Differentiated ES Cells

Differentiated cells were treated with IFN-γ, 1000 U/ml, or mock-treated for 48 h. Compared with undifferentiated ES cells, mock-treated DMSO-differentiated ES cells (Fig. 7a, profile B) expressed reduced levels of ICAM-1, although ICAM-1 remained readily detectable at about 5-fold the fluorescence of the isotype control (Fig. 7a, profile A). After IFN-γ treatment, mean ICAM-1 fluorescence increased significantly to about 10-fold that of the isotype control population (Fig. 7a, profile C), although the fluorescence distribution was more heterogeneous than in the mock-treated population.

MHC-I expression remained undetectable in DMSO-differentiated cells (Fig. 7b, profile B) compared to the isotype control (Fig. 7b, profile A). However, after IFN-γ treatment for 48 h, MHC-I was induced de novo, increasing the mean fluorescence of the population to about 5-fold that of the mock-treated population (Fig. 7b, profile C). Similar to ICAM-1 responses, this response was heterogenous.

The effect of IFN-γ on VCAM-1 and NCAM expression in DMSO-differentiated ES cells, as well as the responses of these cells to WNV infection, was also tested. Table 1 summarizes these results. We found that VCAM-1 remained undetectable in these cells and was not induced by IFN-γ, while NCAM expression remained unaltered after IFN-γ treatment for 48 h. Similarly, WNV failed to up-regulate ICAM-1, VCAM-1, NCAM, or MHC within 24 h of treatment.

DISCUSSION

The expression of Ig superfamily molecules on undifferentiated ES cells during early differentiation in vitro was examined. Using quantitative techniques, it was found that NCAM, ICAM-1, and VCAM-1 antigens were stably expressed at high levels on undifferentiated ES cells in vitro. The levels were higher than in any adult cells we have examined. For example, using the same anti-ICAM-1 antibody (YN1/1.7.4), the levels of ICAM-1 expressed on mouse embryonic fibroblasts and trophoblasts are about 2.5-fold and 1.7-fold those on the isotype controls, respectively [8], and about 2.5-fold the isotype control fluorescence on murine thymocytes (unpublished data). This is in contrast to values 15- to 20-fold the isotype control fluorescence in ES cells reported here. MHC-I, another member of the Ig-CAM family, was not detected on these cells, and this was also the case for MHC-II antigen. These studies were performed with ES cells maintained in their undifferentiated state by culture in media containing LIF. However, a similar pattern of expression was observed when cells were cultured on fibroblast feeder layers, the method originally used to maintain the ES cells in an undifferentiated state. Similar observations of CAM expression from two different ES cell lines and under different culture conditions suggest that expression of these adhesion molecules may be a characteristic of undifferentiated ES cells.

Antibody labeling and flow cytometry can detect specific antigenic determinants of the molecule of interest but do not imply function per se. To confirm that these determinants were associated with a functional molecule, the functionality of ICAM-1 on ES cells was tested. Since the classical function of ICAM-1 is as an adhesion molecule in the adaptive immune response [21], we demonstrated that adhesion of ES cells to activated leukocytes involved ICAM-1. We found that binding was significantly inhibited in the presence of anti-ICAM-1 antibody, which confirmed the functionality of the ICAM-1 molecules detected on the ES cells.

In view of this high level of expression of some of the Ig-CAM molecules on undifferentiated ES cells, the expression of two β2 integrins and a β1 integrin, which act as receptors for CAMs in the adaptive immune response, was examined. Mac-1 and LFA-1 (β2 integrin, receptors for ICAM-1) and VLA-4 (β1 integrin, receptor for VCAM-1) were absent from undifferentiated ES cells. The apparent absence of these receptors raises the question whether Ig-CAMs have a role in adhesion between ES cells. Homophilic interactions between Ig-CAM molecules have been shown to play a role in mutual cellular adhesion in lymphocytes in the immune system [2], and this mechanism may play a role in ES cells. Alternatively, other molecules known to bind ICAM-1, such as CD43 [22], hyaluronan [5], or fibrinogen [23], not investigated in this study, may be present on ES cells. Further studies are required to investigate the role of Ig-CAM molecules in intracellular adhesion of undifferentiated ES cells.

IFN-γ, TNFα, and viral infection can up-regulate the expression of Ig-CAM molecules on many embryonic cell types, including murine midterm trophoblasts and embryonic fibroblasts [8], as well as human embryonic fibroblasts [14]. None of the Ig-CAM molecules tested on undifferentiated ES cells was responsive to the action of these cytokines or viral infection, suggesting that control of CAM expression in these cells may be entirely constitutive. On the other hand, expression of ICAM-1 was significantly reduced and VCAM-1 disappeared almost completely within 12 h of LIF withdrawal. This reduction in expression oc-

curred without detectable changes in morphology or ECMA-7 labeling. High levels of ICAM-1, VCAM-1, and NCAM were also present on undifferentiated ES cells cultured on fibroblast feeder cells in the absence of recombinant LIF, which was the method of choice used to maintain the undifferentiated status of ES cells before LIF became widely available. This suggests that LIF or an equivalent cytokine might be a necessary factor for the expression of Ig-CAMs by undifferentiated ES cells.

One possible explanation for the rapid down-regulation of expression of Ig-CAMs in the absence of LIF is that such down-regulation is an early response to the initiation of differentiation of ES cells. To further examine the effects of differentiation of ES cells on Ig-CAM expression, cells were treated with DMSO for 2 wk. This resulted in loss of their colony-forming morphology, with growth occurring as monolayers and a marked reduction in the number of cells expressing the ECMA-7 antigen. VCAM-1 became undetectable and the expression of both ICAM-1 and NCAM were reduced to about 20% of that observed on undifferentiated stem cells. A further difference between these differentiated cells and undifferentiated ES cells was the observation that ICAM-1 and MHC-I (but not VCAM-1 or NCAM) expression was up-regulated by treatment of the cells with IFN-γ. Their level of expression after IFN-γ treatment was relatively heterogenous, perhaps due to incomplete differentiation from ES cells in this time (ECMA-7 was still expressed in about 42% of these cells) and/or the heterogeneity of differentiated cell lineages produced by DMSO.

These changes in Ig-CAM expression preceding and accompanying visible attributes of differentiation of ES cells suggest that the expression may be linked to the differentiation status of cells. These results do not, however, provide information on whether such changes are necessary in order for differentiation to occur. The extent to which this may be the case warrants investigation. The observation of reduced Ig-CAM expression with differentiation indicates that it would be relevant to test whether it can be used as a marker for the differentiation status and pluripotency of ES cells. Since the ES cells are primary cultures derived from the inner cell mass of mouse blastocysts, it will be important to investigate the status of the Ig-CAM molecules on the inner cell mass and follow its differentiation into the primary germ layers of the embryo.

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REFERENCES

1. Robertson EJ. Embryo-derived stem cell lines. In: Robertson EJ (ed.), Teratocarcinomas and Embryonic Stem Cells—A Practical Approach. Oxford: IRL Press; 1987.
2. Wake A, Tanaka Y, Nakatsuka K, Misago M, Oda S, Morimoto I, Eto S. Calcium-dependent homotypic adhesion through leukocyte function-associated antigen-1/intercellular adhesion molecule-1 induces interleukin-1 and parathyroid hormone-related protein production on adult T-cell leukemia cells in vitro. Blood 1995; 86:2257–2267.
3. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature 1981; 292:154–156.
4. Forrester LM, Bernstein A, Rossant J, Nagy A. Long-term reconstitution of the mouse hematopoietic system by embryonic stem cell-derived fetal liver. Proc Natl Acad Sci USA 1991; 88:7514–7517.
5. Gustafson S, Bjorkman T, Forsberg N, Lind T, Wikstrom T, Lidholt K. Accessible hyaluronan receptors identical to ICAM-1 in mouse mast-cell tumours. Glycoconjugate Journal 1995; 12:350–355.
6. Nakano T, Kodama H, Honjo T. Generation of lymphohematopoietic cells from embryonic stem cells in culture. Science 1994; 265: 1098–1101.
7. Campbell S, Swann HR, Seif MW, Kimber SJ, Aplin JD. Cell adhesion molecules on the oocyte and preimplantation human embryo. Hum Reprod 1995; 10:1571–1578.
8. Tian L, King NJC. IFN-γ induces ICAM-1 on murine midterm trophoblast and enhances susceptibility to specific lysis by paternally-directed allo-immune cytotoxic T cells. Biol Reprod 1994; 51:1164–1172.
9. Pease S, Braghetta P, Gearing D, Grail D, Williams RL. Isolation of embryonic stem (ES) cells in media supplemented with recombinant leukemia inhibitory factor (LIF). Dev Biol 1990; 141:344–352.
10. Williams RL, Hilton DJ, Pease S, Willson TA, Stewart CL, Gearing DP, Wager EF, Metcalf D, Nicola NA, Gough NM. Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature 1988; 336:684–687.
11. King NJC, Sinickas VG, Blanden RV. H-2D and H-2K antigens are independently regulated in mouse embryo fibroblasts. Exp Clin Immunogenet 1985; 2:206–214.
12. King NJC, Kesson AM. Interferon-independent increases in class I major histocompatibility complex antigen expression follow flavivirus infection. J Gen Virol 1988; 69:2535–2543.
13. Taylor WP, Marshall ID. Adaptation studies with Ross River virus: laboratory mice and cell cultures. J Gen Virol 1975; 28:59–72.
14. Shen J, Devery JM, King NJC. Early induction of interferon-independent virus-specific ICAM-1 (CD54) expression by flavivirus in quiescent but not proliferating fibroblasts—implications for virus-host interactions. Virology 1995; 208:437–449.
15. Johnson GD, Davidson RS, McNamee KC, Russel G, Holborrow EJ. Fading of immunofluorescence during microscopy: a study of the phenomenon and its remedy. J Immunol Meth 1982; 55:231–242.
16. Kemler R. Analysis of mouse embryonic cell differentiation. Fortschr Zool 1981; 26:175–181.
17. Bao S, dos Remedios CG, King NJC. Ontogeny of major histocompatibility complex antigen expression on cultured human embryonic skeletal myoblasts. Transplantation 1994; 58:585–591.
18. Gutierre-Ramos JC, Palacios R. In vitro differentiation of embryonic stem cells into lymphocyte precursors are able to generate T and B lymphocytes in vivo. Proc Natl Acad Sci USA 1992; 83:9171–9175.
19. Goodall CA, Curtis ASG, Lang SC. Modulation of adhesion of lymphocytes to murine brain endothelial cells in vitro: relation to class II major histocompatibility complex expression. J Neuroimmunol 1992; 37:9–22.
20. Shen J, Devery JM, King NJC. Adherence status regulates the primary cellular activation responses to the flavivirus, West Nile. Immunology 1995; 84:254–264.
21. Springer TA. Adhesion receptors of the immune system. Nature 1990; 346:425–434.
22. Tiisala S, Majuri ML, Carpen O, Renkonen R. Enhanced ICAM-1-dependent adhesion of myelomonocytic cells expressing increased levels of β2-integrins and CD43. Scand J Immunol 1994; 39:249–256.
23. Hicks RC, Golledge J, Mir-Hasseine R, Powell JT. Vasoactive effects of fibrinogen on saphenous vein. Nature 1996; 376:818–820.