VLA-4–Fibronectin Interaction Is Required for the Terminal Differentiation of Human Bone Marrow Cells Capable of Spontaneous and High Rate Immunoglobulin Secretion

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Summary

Human bone marrow (BM) is a relevant site for immunoglobulin (Ig) generation in vivo. The occurrence of BM cells capable of spontaneous and high rate Ig secretion for 14 d in vitro has been described previously. Accordingly, these cells provide a suitable model for studying terminal B cell maturation within the BM. We have reported recently that these BM cells are not totally differentiated when isolated from the body, as they require inductive signals from adherent stromal BM cells to complete their maturation. Interleukin (IL)-6 produced by these adherent BM cells was identified as one such signal. The present work shows that IL-6 was necessary, but not sufficient, for the induction of BM Ig-secreting cells, since the cytokine was unable to restore missing IgG in nonadherent BM cell cultures. Supernatants (SN) obtained from cultures of stromal adherent BM cells, either freshly isolated or derived from long-term BM culture (LTBMC), restored Ig secretion by nonadherent BM cells, suggesting that additional soluble factors from BM stromal cells were required. Fibronectin (FN) was identified as that factor, as can be deduced from the following findings: (*a*) stromal, but not nonadherent, BM cells constitutively produced FN; (*b*) anti-FN antibodies markedly reduced the IgG secretion in cultures of BM mononuclear cells (BMMC), and blocked the inductive effect of stromal cell SN on nonadherent BM cells, and such a blockade could be reversed by exogenous FN; and (*c*) finally, although neither IL-6 nor FN alone exerted any effect, the combination of both factors induced optimal Ig secretion by nonadherent BM cells. Furthermore, VLA-4 molecules seemed to be the FN receptor that was active in this culture system, as indicated by: (*a*) BM Ig-secreting cells exhibited the phenotype VLA-4^+ VLA-5^-; (*b*) mAbs directed to VLA-4 (anti-CD29 and anti-CD49d), but not those directed to other adhesion molecules, inhibited Ig secretion by BMMC cultures, and this effect was reversed by FN; (*c*) the inductive role of the entire FN molecule could be replaced by a fragment containing the CS-1 region, but not by a fragment containing the RGDS sequence; and (*d*) only mAbs anti-CD49d capable of blocking VLA-4–FN interaction inhibited induction by either the FN or the CS-1–containing fragment of FN. These results suggest that, in addition to IL-6, the interaction of FN produced by stromal BM cells with VLA-4 molecules present on the surface of BM producers is critical for the latter cells to differentiate into the prolonged and high rate Ig-secreting stage characteristic of these cells. Therefore, cells from the marrow microenvironment might contribute to the terminal maturation of Ig-secreting BM cells in vivo.

In adult life, the mammalian bone marrow (BM)[1] is the main reservoir for high rate Ig-secreting B lymphocytes (1, 2). The origin of these cells has not been fully clarified. At least in part, they appear to be generated in distant lymphoid tissues upon exposure to antigens, and, after a short period of maturation, migrate into the BM in a "pro-plasma cell" state (3–6). Accordingly, the BM becomes an important site for antibody and serum Ig formation (1, 2).

BM B cells capable of spontaneous and high rate Ig secretion in vitro have been described in several species (5, 7). In humans, cells of this kind are present at low frequencies (10^{-3}–10^{-4} in BM mononuclear cell [BMMC] fractions), and still produce considerable quantities of Ig (10^8 molecules/cell per hour) over a period of 14 d without requiring

[1] *Abbreviations used in this paper:* BM, bone marrow; BMMC, bone marrow mononuclear cells; Cx, cycloheximide; ECM, extracellular matrix; FN, fibronectin; LTBMC, long-term BM culture; MPA, mycophenolic acid; OH-U, hydroxyurea; SN, supernatant.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/92/06/1739/09 $2.00
Volume 175 June 1992 1739–1747

intentional stimulation (7–9). This subset consists of large nonproliferating cells that exhibit the phenotype $CD20^-$ $CD19^{\pm}$ $CD38^+$ (7–9). Furthermore, the presence of these cells has been clearly connected with in vivo-induced antibody responses (10, 11), and there is evidence suggesting that they could account for most of serum Ig generation (8). Altogether, these features indicate that human BM spontaneous Ig-secreting cells have reached an advanced stage of maturation in vivo. As such, they appear particularly adequate for analyzing the regulation of terminal B cell differentiation within the BM.

In a previous report (12), we have shown that these cells are not totally differentiated, when isolated from the body. Adherent BM stromal cells, either freshly isolated or derived from long-term BM culture (LTBMC), provided the auxiliary signals necessary for these BM B cells to proceed into the high rate and prolonged Ig-secreting stage. IL-6 production by BM stromal cells seems to be essential in this process.

The present study shows that IL-6 is necessary but not sufficient for the terminal maturation of BM Ig-secreting cells, since an additional factor produced by BM stromal cells is required. This factor was found to be the well-known component of the serum and of the extracellular matrix (ECM) fibronectin (FN). Additionally, VLA-4 molecules are shown to be present on the surface of the BM Ig-secreting cells, and to function as FN receptors on such cells. These results indicate that VLA-4–FN interaction plays a relevant role in the differentiation of human BM cells capable of high rate Ig secretion.

Materials and Methods

Materials. Hydroxyurea (OH-U) and cycloheximide (Cx) were purchased from Calbiochem Corp. (San Diego, CA). Purified and PE-conjugated OKT10 (CD38) mAb was obtained from Ortho Diagnostic Systems (Raritan, NJ). Rabbit anti-human FN antibody was provided by Dako Corp. (Glostrup, Denmark). Anti-human FN mAb and Insulin-Transferrin-Sodium Selenite Supplement used in serum-free cultures were from Boehringer Mannheim (Mannheim, Germany). Goat anti-human IgG and peroxidase-conjugated goat $F(ab')_2$ anti-human IgG used in the ELISA sandwich for IgG detection, and peroxidase-conjugated goat $F(ab')_2$ anti-rabbit IgG used in the sandwich ELISA for FN were from Tago Inc. (Burlingame, CA). FITC-conjugated goat $F(ab')_2$ anti-human Ig and anti-mouse IgG used in direct and indirect immunofluorescence procedures, respectively, were provided by Kallestad Diagnostics (Austin, TX). Magnetizable beads conjugated with goat anti-mouse Ig antibodies and the magnetic particle concentrator (MPC™ 1) used in cellular selection techniques were obtained from Dynal (Oslo, Norway). M24-AR Dynatech microtiter plates were used in the ELISA test for IgG and FN quantitation (Dynatech Laboratories Ltd., Sussex, England). The 96- and 24-well flat-bottomed plates, the 25 cm^2 Roux flasks and the Transwell chambers (catalog no. 3413; Costar Corp., Cambridge, MA) used for cell cultures, as well as the polystyrene Petri dishes used for the panning procedure and adherence separation were from Costar Corp. (Cambridge, MA). Recombinant human IL-6 was provided by Genzyme Corp. (Boston, MA). Gelatin and laminin were purchased from Sigma Chemical Co. (St. Louis, MO). $[^3H]$TdR with a specific activity of 5 Ci/mmol was purchased from Amersham International (Amersham, Bucks, UK).

mAb against Adhesion Molecules. Purified and FITC-conjugated mAb IOP49e (anti-CD49e, VLA α_5), IOL44 (anti-CD44), and IOL54 (anti-CD54, ICAM1) were provided by Immunotech S.A. (Luminy, France). mAb BBA6 (anti-VCAM 1) was provided by British Bio-technology Ltd. (Oxan, UK). mAb TS2/16 (anti-CD29, VLA β_1), mAb HP2/1 and HP1/3 (anti-CD49d, VLA α_4), mAb TP1/40 (anti-CD11a, LFA1), and mAb TS2/7 (anti-CD49a, VLA α_1) (13, 14) were the gift of Dr. F. Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain). mAb P1D6 (anti-CD49e, VLA α_5) (15) was the gift of Dr. E. Wyner (Cytel Corp., La Jolla, CA).

BMMC Preparation and Purification. BM cells were obtained from ribs removed during thoracotomy from patients with localized lung tumors. The patients were otherwise healthy. None had received steroids, cytostatic drugs, or radiotherapy, and their ages ranged from 34 to 58 yr. After cutting the ribs into pieces, BM cells were washed out by vigorous pipetting with HBSS, and were washed twice in the same medium. BMMC were prepared by Ficoll-Hypaque density centrifugation. Fresh adherent BM cells were obtained after incubation of BMMC at 2×10^6 cells/ml on plastic petri dishes for 3 h, as previously reported (16). This population accounted for an average of 20% of the recovered cells. The composition of this freshly isolated BM adherent population has been previously reported (17), and consisted of mixed fibroblasts, myeloid cells, macrophages, endothelial cells, and lymphoid cells. Nonadherent cells were further depleted of residual adherent cells by magnetic removal of cells that phagocytosed carbonyl iron particles (18). Cytocentrifugal preparations of unfractionated, adherent, and nonadherent BM cells were fixed in acetone, and the proportion of cells containing cytoplasmic Ig was estimated by a direct immunofluorescence technique (19). BM nonadherent cells bearing and lacking VLA-4 and -5 molecules on their surface were obtained by treatment with specific mAbs and immuno-magnetizable beads conjugated with goat anti-mouse Ig antibodies, as reported (20). In brief, nonadherent BM cells at 10^7 cell/ml were incubated with 1 μg of HP2/1 (anti-CD49d) or IOP49e (anti-CD49e) mAbs for 30 min at 4°C, washed once, and incubated with goat anti-mouse Ig-coated beads (at a bead/cell ratio of 3:1) for 30 min at 4°C. Cells were then diluted five times in cold PBS, and those which bound magnetizable beads were retained on the tube wall by a magnetic particle concentrator. Negative and positive cells for both markers were recovered, washed twice, and cultured. The efficiency of the separation method was verified by indirect immunofluorescence and flow cytometry analysis in an Epics Profile cytometer (Coulter Corp., Hialeah, FL). $VLA\text{-}4^+$ and -5^+ populations contained more than 90% positive cells for the corresponding marker, whereas <5% positive cells were present in the negatively selected cell fractions $VLA\text{-}4^-$ and $VLA\text{-}5^-$.

In some experiments, BM cells capable of spontaneous Ig secretion were purified. To this end, adherent BM cells were removed as indicated above, and the nonadherent cells were then depleted of T lymphocytes by a previously described rosette technique (21). Non-T nonadherent BM cells were further fractionated according to the presence of CD38 molecules on their membrane by a previously described panning technique (9). Positively selected populations ($CD38^+$) commonly exhibited more than 85% positive cells for the CD38 marker and will be referred to as BM $CD38^+$ cells. Spontaneous Ig-secreting cells were enriched in the purified BM $CD38^+$ population by an average of five times (9).

FN and FN Fragments. Human plasma FN was the generous gift of Drs. B. Horowitz and R. Shulman (New York Blood Center, New York). FN fragments of 80 and 38 kD were prepared by trypsin

digestion (1/200, wt/wt, 90 min, 37°C), as previously described (22, 23). The 80-kD fragment contains the RGDS sequence (23), and the 38 kD fragment contains the entire HepII domain and most of the IIICS region of FN, including the CS-1 sequence (22). ECM proteins including FN, FN fragments, gelatin, and laminin were assessed in soluble as well as in fixed forms, as indicated. To obtain this latter form, microtiter culture wells were incubated with the proteins at indicated concentrations in 100 μl of PBS for 2 h at room temperature, followed by extensive washing with PBS and a new incubation with 1% BSA-PBS. The plates were used the same day.

Preparation of Stromal Cells Derived from LTBMC. Stromal cells were derived from LTBMC, according to the technique described by Kierney and Dorshkind (24). Briefly, 5 ml of a suspension of fresh BMMC at 2 × 10^6 cells/ml were cultured in 25 cm^2 Roux flasks, in a culture medium containing 20% FCS, and half of the supernatant (SN) was replaced weekly with fresh medium. To eliminate the remaining hematopoietic cells, all of the medium was removed after 2 wk, and the cells were cultured in fresh medium containing mycophenolic acid (MPA) at 5 μg/ml for 3 d. After this period, the cells were again incubated in fresh medium without MPA. This treatment was repeated on days 20 and 28. Subsequently, the cultures were continued by weekly replacement of half of the medium with fresh medium. At confluence, cultures were trypsinized and subcultured as needed. Stromal cells maintained for 60–120 days were used in this work. After 60 d of culture, LTBMC-derived stromal cells exhibited the appearance and phenotype of fibroblasts (12).

Cell Culture. BMMC and BM cell fractions obtained by magnetic selection techniques were cultured at a concentration of 10^6 cells/ml. Adherent and nonadherent BM cells were cultured at 0.2 × 10^6 cells/ml and at 0.8 × 10^6 cells/ml, respectively. All the cultures were set up in 96-well flat-bottomed plates in a final vol of 0.25 ml for 14 d, unless otherwise indicated. The cultures were incubated at 37°C with 5% CO_2 in a culture medium consisting of RPMI 1640 supplemented with 10% FCS, L-glutamine (10 mM) and gentamycin (0.05 mg/ml). The serum-free medium used in this work was identical, except for the substitution of a supplement consisting of transferrin (5 μg/ml), insulin (5 μg/ml), and sodium selenite (5 ng/ml) for FCS. At the end of the culture period, cell-free supernatants were obtained by centrifugation and were stored at −20°C until ELISA testing. DNA synthesis, measured as [^{3}H]TdR-uptake, was evaluated in certain cultures by pulsing them with 1 μCi of [^{3}H]TdR during the last 16 h of culture. Cells were then harvested onto glass fiber filters and counted by liquid scintillation spectrometry. To obtain conditioned SN from BM stromal cells, freshly isolated (2 × 10^5 cells/ml), as well as LTBMC-derived (2 × 10^4 cells/ml) adherent cells were cultured, and the culture media were recovered after 3 d and frozen until use.

Solid Phase ELISA for IgG, IL-6, and FN Determination. The quantitative ELISA used to measure the IgG secreted into the culture SN was performed in microtiter plates, as described by de la Concha et al. (25). IL-6 was also determined in the same SN by using an ELISA technique (InterTest-6™ ELISA kit, Genzyme Corp., Boston, MA). FN present in culture supernatants was determined by a sandwich ELISA in microtiter plates, as follows: (*a*) wells were coated with 400 ng of anti-FN mAb in 200 μl of PBS for 16 h at room temperature; (*b*) free binding sites were blocked by incubating the wells with 1% BSA-PBS for 1 h; (*c*) purified FN and suitably diluted culture supernatants were incubated for 2 h; (*d*) 200 μl of polyclonal rabbit anti-human FN antibodies diluted in 1% BSA-PBS at 1/4,000 were added to each well and incubated for 2 h; and (*e*) finally, the wells were incubated with peroxidase-conjugated goat anti-rabbit Ig antibodies at 1/1,000 dilution in 1% BSA-PBS for 30 min, and revealed with α-phenylendiamine. The standard curve for FN was linear in the range of 2–50 ng.

Results

BM High Rate IgG-secreting Cells Require the Cooperation of BM Stromal Cells. We have previously reported that the majority of the BM cells capable of spontaneous and high rate Ig secretion are not totally differentiated when isolated from the body, but that they still require the presence of inductive factor(s) provided by adherent BM stromal cells. IL-6 was identified as such a factor (12). Fig. 1 summarizes these observations: Fig. 1 *A* shows that unstimulated BMMC cultures produced IgG in a linear fashion over 2 wk; adherent BM cells did not secrete any IgG; nonadherent BM cells showed a low and short-term curve of IgG production, in spite of the fact that Ig-secreting cells, determined either as $CD38^{+bright}$ cells or as cells containing cytoplasmic Ig, were retained in this cell fraction; the coculture of the two latter populations restored IgG secretion to the level of unfractionated BMMC, and this effect could be reversed by the addition of blocking anti-IL-6 antibodies. Fig. 1 *B* shows that the BM adherent cells were responsible for the endogenous IL-6 synthesis in these cultures. A similar IL-6–mediated effect was obtained when either nonadherent BM cells or purified $CD38^+$ cells were cocultured with LTBMC-derived fibroblasts (12, and data not shown). Despite the important role demonstrated for IL-6 in this culture system, Fig. 2 shows that the cytokine alone was incapable of inducing IgG secretion in BM cells depleted of adherent cells, suggesting that additional signals provided by BM adherent stromal cells were needed for the high rate Ig secretion by BM cells to occur.

Nature of the Signals Derived from BM Stromal Cells. Subsequently, the possible requirement for signal(s) conveyed

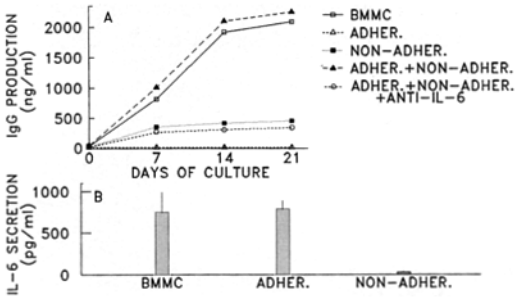

Figure 1. Role of IL-6 and BM adherent cells in spontaneous IgG secretion by cultured BMMC. (*A*) BMMC (10^6 cells/ml), adherent (*ADHER.*) BM cells (0.2 × 10^6 cells/ml), nonadherent (*NON-ADHER.*) BM cells (0.8 × 10^6 cells/ml), and a combination of the two latter populations, were cultured, and the IgG secreted to the supernatant at different times was measured. Anti-IL-6 mAb was added to certain cultures at a final dilution of 1/2,500. Results of one of four similar experiments are shown, and represent the mean of duplicate cultures. (*B*) The production of IL-6 by the same BM cell populations was determined after 3 d of culture. Results represent the mean ± SEM of five experiments.

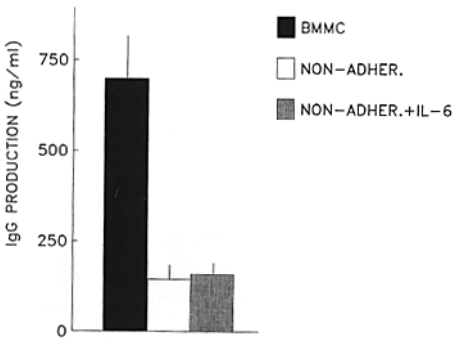

Figure 2. The effect of IL-6 on the IgG secretion by nonadherent BM cells. BMMC and nonadherent (*NON-ADHER.*) BM cells were cultured for 14 d and the IgG secreted to the supernatants was determined. IL-6 (1 ng/ml) was added to indicated cultures. Results represent the mean ± SEM of eight experiments.

through direct contact between adherent BM stromal cells and and BM Ig-secreting cells was investigated in two different approaches. First, since the adhesion molecules are involved in many examples of processes mediated by cell to cell contact, the effect of adding mAb directed against such molecules was explored in BMMC cultures. As shown in Fig. 3, only mAb directed to the VLA-4 heterodimer (CD29 CD49d) markedly inhibited IgG secretion in these cultures. Secondly, BM stromal cells and nonadherent BM cells were cocultured either together (cellular contact present) or separated in the two chambers of a Transwell culture system (cellular contact prevented). Table 1 shows that separated and unseparated cocultures produced similar quantities of IgG, ruling out the necessity for close cellular interactions in the present system. This fact was confirmed by the observation that SN obtained from cultures of freshly isolated adherent BM cells, as well as of LTBMC-derived stromal cells, were capable of restoring IgG secretion by nonadherent BM cells (Fig. 4). These results indicated two apparently contradictory facts: the integrin molecule VLA-4 seemed to be involved in the regulation of BM cells capable of high rate IgG secretion; and the inductive role of stromal cells on BM high rate IgG-secreting cells was due to soluble factor(s) produced by the former cells.

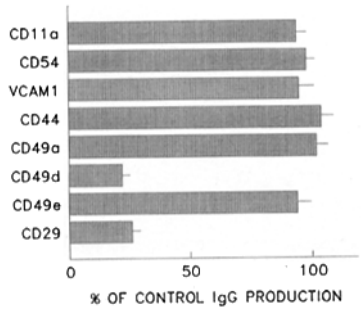

Figure 3. Effect of the addition of mAb directed to a variety of adhesion molecules. BMMC were cultured for 14 d in the presence and absence of a concentration of 2 μg/ml of indicated mAbs, and the IgG secretion was determined. The values were expressed as a percentage of untreated control cultures. Results represent the mean ± SEM of seven experiments. Control IgG secretion in these experiments was 712 ± 187 ng/ml.

Table 1. *Requirement for Cellular Contact in the Inductive Effect of BM Stromal Cells on BM IgG-secreting Cells*

	Cellular contact	
Exp. no.	Present	Prevented
1‡	992*	960
2‡	400	336
3§	2,150	2,450
4§	900	1,000

* Results represent the mean IgG production (ng/ml) in duplicate cultures.
‡ 2 × 10^5 freshly-isolated BM adherent cells were cocultured with 8 × 10^5 BM nonadherent cells either together (contact present) or in the two separate chambers of a Transwell culture system (contact prevented). 1-ml cultures were set up in 24-well plates for 14 d.
§ 2 × 10^4 LTBMC-derived stromal cells were cocultured with 2 × 10^5 BM $CD38^+$ cells, as described above.

The Inductive Effect of BM Stromal Cells Is Mediated by IL-6 plus FN. Recent reports have clearly demonstrated that the well-defined component of the serum and of ECM FN is a ligand for VLA-4 (26–28). In addition, BM cells can secrete FN (29). Therefore, the possibility that FN was involved in the regulation of BM high rate IgG-secreting cells was investigated. First, FN production by BM cells in the present system was tested using FCS-free cultures. Fig. 5 *A* shows that freshly isolated BM adherent cells, as well as LTBMC-derived stromal cells spontaneously and actively produced large amounts of FN in 3-d cultures. BM nonadherent cells did not produce any FN. Kinetic studies revealed that an average of 70% of the FN secreted by adherent BM cells occurred in the first 4 h of culture (data not shown). Moreover, Fig. 5 *B* shows that the addition of anti-FN antibodies to BMMC cultures drastically reduced their subsequent IgG secretion, and this effect could be reversed by exogenous FN. The inhi-

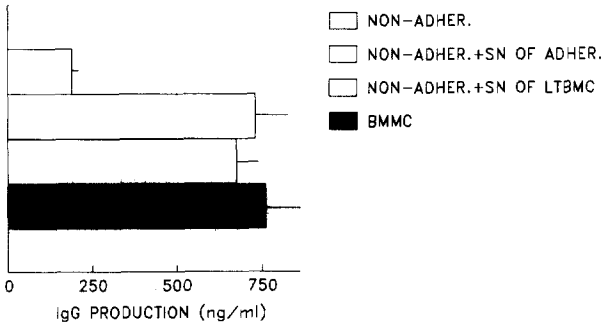

Figure 4. Effect on BM IgG-secreting cells of the conditioned SN obtained from cultures of adherent BM cells. BMMC and nonadherent (*NON-ADHER.*) BM cells were cultured for 14 d in the presence and absence of 50% SN obtained from cultures of freshly isolated BM adherent cells (*SN OF ADHER.*) and LTBMC-derived fibroblasts, and the secreted IgG was evaluated. Results represent the mean ± SEM of eight experiments.

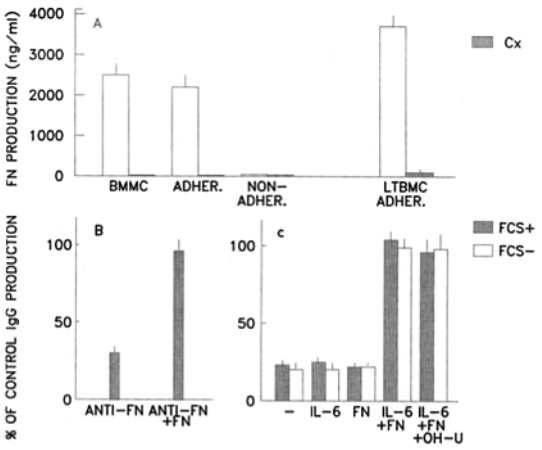

Figure 5. Role of FN on BM spontaneous IgG-secreting cells. (*A*) FN secretion by BM cells. BMMC, adherent (*ADHER.*) and nonadherent (*NON-ADHER.*) BM cells, and LTBMC-derived fibroblasts were cultured for three days in serum-free medium, and the quantity of FN released to the supernatant was determined by ELISA. Cycloheximide (Cx; 10 μg/ml) was added to certain cultures. Results represent the mean ± SEM of four experiments. (*B*) BMMC were cultured for 14 d in the presence and absence of FN (15 μg/ml) and of anti-FN polyclonal Ab at a dilution of 1/50, and IgG secretion was measured. (*C*) Nonadherent BM cells were cultured either in FCS-containing (FCS$^+$; *filled boxes*) or serum-free (FCS$^-$; *open boxes*) medium for 14 d in the presence and absence of IL-6 (1 ng/ml), FN (15 μg/ml), and hydroxyurea (OH-U; 0.5 mM), and the IgG secreted to the supernatant was evaluated. Values were expressed as a percentage of control IgG secretion. Results represent the mean ± SEM of nine and seven experiments for B and C, respectively. The control IgG production in the corresponding experiments was 547 ± 213 ng/ml, and 937 ± 358 ng/ml for B and C, respectively.

bition mediated by anti-FN antibodies was concentration dependent, and the effect reached a plateau at 1/50 dilution of the antibody, sparing some 10–20% of the IgG secretion. Taken together, these observations strongly suggested that, in addition to IL-6, the endogenous generation of FN by BM stromal cells might be important in the regulation of BM high rate IgG-secreting cells. Therefore, the effect of adding exogenous FN on cultures of nonadherent BM cells was assessed. As shown in Fig. 5 *C*, neither optimal quantities of IL-6 (1 ng/ml), nor FN used at 0.1–100 μg/ml (only data of adding 15 μg/ml of FN are shown) was capable of inducing IgG secretion in nonadherent BM cell cultures by itself. The combination of both factors, however, restored complete IgG production. This effect was similarly observed in the presence (*filled boxes*) and absence (*open boxes*) of FCS, clearly indicating that the FN present in the FCS did not act in the system. The addition of FN in either soluble or fixed form was efficient in inducing optimal IgG secretion in cultures of nonadherent BM cells supplemented with IL-6. This FN-mediated induction was concentration dependent, and the effect reached a plateau at 5 μg/cm^2 and 15 μg/ml for the fixed and soluble form, respectively. Neither laminin nor gelatin used in a wide range of concentrations was capable of restoring the IgG secretion in similar cultures. It should also be noted in Fig. 5 *C* that the inductive effect of IL-6 plus FN on IgG secretion was not altered by the addition of the DNA synthesis inhibitor hydroxyurea (OH-U), used at a concentration capable of reducing over 90% of the PHA-induced proliferative response by blood lymphocytes. In line with this latter observation, the addition of IL-6 plus FN to these cultures did not induce DNA synthesis, detected as [^{3}H]TdR-uptake. Furthermore, the inductive effect of BM stromal cell SN on IgG secretion by nonadherent BM cells was equally inhibited by the addition of either anti-FN or anti-IL-6 antibodies. The inhibition mediated by these antibodies could be reversed by adding exogenous FN and IL-6, respectively (data not shown).

Table 2. *Presence of VLA-4 and -5 Molecules on the Surface of BM Cells Capable of High Rate IgG Secretion*

Exp. no.	Unseparated cells*	IgG secretion VLA-4$^+$	VLA-4$^-$	VLA-5$^+$	VLA-5$^-$
		ng/ml			
1	370‡	530	42	<5	320
2	720	880	30	<5	1,040

* Unseparated cells and cell fractions of BM nonadherent cells were cultured at 10^6 cells/ml for 14 d. IL-6 (1 ng/ml) and FN (15 μg/ml) were added to all cultures. No attempt was made to detach the magnetic beads from the cell surface in the positive fractions; however, the separation procedure did not seem to affect IgG secretion.
‡ Results represent the mean of duplicate cultures.

BM Cells Capable of Spontaneous and High Rate IgG Secretion Exhibit the FN Receptor VLA-4. To further substantiate the involvement of FN in the induction of Ig secretion by the BM cell subset under study, the expression of the two well-known FN receptors VLA-4 and -5 (26–28, 30, 31) on these cells was examined. First, BM cells were labeled for the CD38 antigen (OKT 10-PE mAb) and for either VLA-4 (HP2/1 mAb-FITC) or -5 (IOP49e mAb-FITC) molecules, and flow cytometry analysis showed that all the cells expressing high levels of CD38 antigens, which include the BM-spontaneous Ig-secreting cells (9), also coexpressed high levels of VLA-4, but not -5 molecules (data not shown). To confirm this finding, nonadherent BM cells were separated into positive and negative cell fractions for these latter markers, and the IgG secretion by unseparated and different separated cell fractions (VLA-4$^+$, VLA-4$^-$, VLA-5$^+$, and VLA-5$^-$) was evaluated after 14 d of culture in the presence of IL-6 plus FN. The results contained in Table 2 indicated that BM IgG-secreting cells showed the phenotype VLA-4$^+$ VLA-5$^-$.

VLA-4–FN Interaction Is Essential for the Function of BM Cells Capable of High Rate Ig Secretion. The above results suggested that the interaction of FN with the cellular receptor VLA-4 was involved in the regulation of BM cell Ig secretion. To confirm this, the effect of FN on the inhibition of IgG secretion mediated by anti-VLA-4 mAb (Fig. 3) was examined. Fig. 6 *A* shows that the inhibitory effect of HP2/1

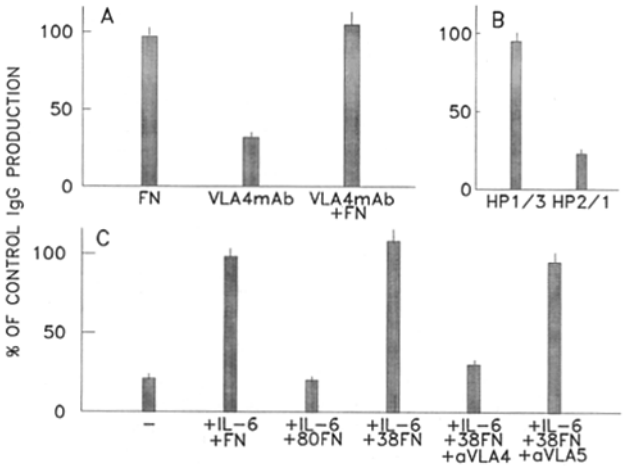

Figure 6. Role of VLA-4–FN interaction in the IgG secretion by BM cells. (*A*) BMMC were cultured for 14 d in the presence and absence of anti-CD49d mAb HP2/1 (2 μg/ml) and FN (15 μg/ml), and the IgG secreted to the SN was measured. (*B*) Similar cultures were set up in the presence and absence of 2 μg/ml of the indicated anti-CD49d mAbs, and the IgG secretion was determined. (*C*) Nonadherent BM cells were cultured in the presence and absence of IL-6 (1 ng/ml), FN (5 μg/cm²), 80 kD FN fragment (80FN; 5 μg/cm²), 38 kD FN fragment (38FN; 5 μg/cm²), anti-CD49d mAb HP2/1 (αVLA4; 2 μg/ml), and anti-CD49e mAb P1D6 (αVLA5; 2 μg/ml), and IgG secreted to the SN was evaluated. Values were expressed as a percentage of the control IgG production in untreated BMMC cultures. Results are expressed as the mean ± SEM. of five, four, and four experiments for *A*, *B*, and *C*, respectively. Control IgG secretion in these experiments was 460 ± 156, 725 ± 232, and 812 ± 380 ng/ml for A, B, and C, respectively.

(anti-CD49d) mAb on BMMC IgG secretion could be reversed by the addition of exogenous FN. Furthermore, as shown in Fig. 6 *B*, the inhibitory effect of anti-CD49d mAb was only observed with the mAb HP2/1, which has been shown to block the FN-binding site of VLA-4 molecules, but not with the mAb HP1/3, which does not interfere with FN VLA-4 recognition (32).

To establish the specificity of this FN-mediated phenomenon more clearly, two different fragments of the FN molecule were prepared, one of 38 kD containing the CS-1 region, and another of 80 kD comprising the RGDS sequence (22, 23), and their inductive effects on IgG secretion by nonadherent BM cells were evaluated. As shown in Fig. 6 *C*, the 38-kD fragment fully restored IgG secretion by IL-6–supplemented nonadherent BM cell cultures. This effect could be inhibited by anti-VLA-4 mAb, but not by anti-VLA-5 mAb. In contrast, the 80-kD fragment exerted no effect.

Similar results were found when IgA and IgM were evaluated in these cultures (data not shown).

Discussion

It is now well established that the marrow microenvironment plays an essential role in the ontogenic development of many cell types, including lymphocytes (33, 34). Evidence accumulated in recent years has greatly increased our knowledge about the cellular and molecular mechanisms by which this supportive effect of BM is accomplished. Thus, a variety of BM stromal cells capable of producing cytokines and ECM proteins have been involved in many aspects of BM hemopoiesis (35). Despite the fact that the majority of B lymphocytes capable of high rate Ig secretion are located in this organ (1, 2), little is known about their interaction with marrow cells. Namely, it remains to be elucidated whether the BM is a storage organ for B lymphocytes that have reached full maturity, or whether it influences the terminal differentiation of the Ig-secreting cells.

Human BM cells capable of spontaneous and high rate Ig secretion in vitro appear to be a relevant model for analyzing the regulation of final B cell maturation in the BM. It has been previously shown that IL-6 production by BM stromal cells was required for most of the Ig secretion occurring in this system, and this suggested a regulatory role for cells of the marrow microenvironment (12). Present results indicate that an additional factor synthesized by BM stromal cells was also needed, since exogenous IL-6 was unable to induce Ig secretion in the absence of adherent BM cells. This was confirmed by the finding that BM stromal cell SN restored Ig secretion by nonadherent BM cell cultures. FN was identified as this additional factor, as can be deduced from the following observations: (*a*) as previously reported (29), and also demonstrated here, BM stromal cells constitutively produced FN; (*b*) anti-FN antibodies markedly reduced the IgG secretion in BMMC cultures and blocked the inductive effect of stromal cell SN on nonadherent BM cells, and this blockade could be reversed by exogenous FN; and (*c*) finally, although neither IL-6 nor FN alone exerted any effect, the combination of both factors induced optimal Ig secretion by BM nonadherent cells. These findings demonstrate that FN and IL-6 each alone were necessary, but not sufficient, factors, and that the coordinated activity of both was required for optimal induction of BM high rate Ig-secreting cells. Since FN rapidly adheres onto culture surfaces, the experiments described above did not clarify whether this ECM molecule might act in a soluble nonfixed form in the present system. In contrast to the well-established role of IL-6 as a B cell differentiation factor (36–39), FN had not been clearly related to this activity.

It should be noted that a minor part of BMMC spontaneous Ig secretion seemed to be independent of IL-6 and FN, since 10–25% of this activity remained in the presence of saturating quantities of anti–IL-6 (12) and anti-FN blocking antibodies. This might reflect the existence of cells that had already received the inductive signal in vivo.

Three different FN receptors have been identified in the VLA family of integrins: VLA-3 (CD29 C49c), VLA-4 (CD29 CD49d), and VLA-5 (CD29 CD49e) (23, 26–28, 30, 31). VLA-3 is mainly expressed on most adherent cells, and binds FN at an undetermined site (40). VLA-4 and -5 are widely expressed in the lymphoid system (41). VLA-5 recognizes the RGDS sequence located in the central region of all FN molecules (30, 31). VLA-4 binds to the CS-1 region present in FN molecules containing the alternatively spliced segment

known as IIICS domain (26–28). Present data strongly suggest that VLA-4 molecules function as FN receptors in this system. This is based on the following facts: (*a*) mAbs directed to CD29 and CD49d, but not those directed to other adhesion molecules, inhibited Ig secretion in BMMC cultures, and this effect was reversed by FN; (*b*) the inductive role of the entire FN molecule could be replaced by a fragment containing the CS-1 region, but not by a fragment containing the RGDS sequence; and (*c*) only mAbs anti-CD49d capable of blocking VLA-4 FN interaction (32) inhibited induction by either FN or the CS-1-containing fragment of FN. Therefore, VLA-4 FN interaction plays a critical role in the present culture system, and, along with IL-6, appears to fulfill all the signaling requirements for induction of high rate Ig secretion by BM cells.

FN contained in the FCS was found to be inefficient in restoring Ig secretion by nonadherent BM cell cultures supplemented with IL-6. The reason for this is unknown. An explanation could be the reported structural differences, notably in carbohydrate contents, between plasma and fetal (placental) FN (42, 43), which may somehow affect the interaction with the cell surface.

Interactions between cells and ECM components seem to control important biological processes such as cell migration, embryogenesis, tumor metastasis, and wound healing. Specifically, FN–VLA-4 interaction has been implicated in the promotion of cell anchorage and migration of hematopoietic precursors, activated B and T lymphocytes, and NK cells (44–47), and in the proliferation of T cells (48–50). FN has also been demonstrated to supply differentiation signals in several cell systems (44, 51–54), and VLA-4 appeared to be the cellular FN receptor in some of these instances (44, 54). Data in the present paper indicate that the inductive effect of FN plus IL-6 on BM Ig-secreting cells occurred in the apparent absence of cell growth, since DNA synthesis was not detectable in these cultures and Ig secretion was not affected by DNA synthesis inhibition. Therefore, IL-6 plus FN conveys a differentiation signal upon the receival of which the BM producers proceed into the prolonged and high rate Ig-secreting stage characteristic of these cells.

The mechanism(s) by which this maturative event is triggered by IL-6 plus FN is unknown. The finding that BM Ig-secreting cells exhibited VLA-4 molecules, and that the requirement for VLA-4–FN recognition was evidenced in BM CD 38^+ cell cultures supports the view that this latter interaction takes place on the surface of the secreting cell. In addition, the fact that neither of the two factors has any effect alone could indicate that IL-6 plus FN delivers an integral signal to these cells, perhaps in a manner similar to what has been proposed for fibroblast growth regulation by basic fibroblast growth factor, cellular and ECM heparan-sulfate, and the polypeptidic cellular receptor (55).

B cell differentiation has been traditionally understood as a T cell–dependent event. The present subset of Ig-secreting cells, which probably represents a relevant stage in the sequence of B cell differentiation in vivo, seemed to undergo terminal maturation under the influence of cells of the marrow microenvironment and their products. This observation suggests that in vivo differentiation of high rate Ig-secreting lymphocytes within the BM could be primarily mediated by stromal cells rather than by T cells. Such a notion is compatible with the in vivo pattern of homing and distribution of plasmablasts and plasma cells in the BM, since they are mostly found at the adventitial space of the BM arterial capillaries, juxtaposed to the FN-rich vascular basal membrane, in close vicinity to stromal, but not T cells (56, and data not shown).

The authors wish to thank Pilar Salillas, Francine Doat, and Consuelo Salgado for excellent technical assistance, and Dr. Bootello for continuous encouragement.

This work was supported by grant 90/255 from Fondo de Investigaciones de la Seguridad Social (FISSS) of Spain.

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Received for publication 6 January 1992 and in revised form 26 February 1992.

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