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A Ahmad; ... et. al

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Binding of the Epstein-Barr Virus to Human Platelets Causes the Release of Transforming Growth Factor-β[1]

Ali Ahmad[2] and José Menezes

Human platelets bear on their surface complement receptor type II (CR2), which is also the receptor for the EBV. Although the cross-linking of these receptors causes activation and aggregation of platelets, no immunologic consequence of the potential binding of EBV to these receptors on human platelets has ever been described. We report here that binding of EBV to human platelets causes the release of TGF-β from the latter. Both infectious and UV-inactivated noninfectious viral particles can mediate this release. Anti-CR2 mAb OKB7, which blocks the binding of EBV to CR2, also blocks the EBV-mediated release of TGF-β. Furthermore, platelets recovered from the initial incubation no longer release TGF-β upon subsequent incubation with EBV. Since TGF-β is a potent immunosuppressive agent, its release from platelets upon binding of EBV may play a role in the pathogenesis of EBV-associated diseases. *The Journal of Immunology,* **1997, 159: 3984–3988.**

Platelets or thrombocytes constitute the most numerous physical entities of blood after RBC. Derived from the precursor cells, megakaryocytes, they circulate in blood for 7 to 10 days before eventually being removed from circulation by cells of the reticulo-endothelial system (reviewed in Ref. 1). They contain no nucleus, and little or no protein synthesis occurs in them. Nevertheless, upon appropriate stimulation they are capable of secreting numerous preformed substances, e.g., platelet-derived growth factor, platelet-activating factor-4, TGF-β1, etc. (1, 2). In fact, platelets are the richest source of TGF-β in the human body (3). These substances are stored in their secretory granules. Upon activation, platelets can also perform the de novo synthesis of several substances, e.g. thromboxane A_2, by lipid hydrolysis (1). The role of platelets in hemostasis and thrombosis is well documented; however, several studies indicate that platelets may play a role in the host defense against pathogens and malignancy by cytolytic mechanisms and may also contribute to their pathogenesis (4–8). Furthermore, activated platelets may express on their surface and/or release substances that can potentially modulate an ongoing immune response (9, 10). Despite these capabilities, our knowledge about their role in the pathophysiology of infectious diseases is rudimentary.

Platelets bear on their surface receptors for the C_3d fragment of complement (CR2),[3] which also serve as receptor for EBV (11–14). EBV is a ubiquitous human herpesvirus that is known to cause infectious mononucleosis and is associated with several other human lymphoproliferative and autoimmune disorders, notably endemic Burkitt's lymphoma, undifferentiated nasopharyngeal carcinoma, Hodgkin's lymphoma, Sjögren's syndrome, hairy cell leukoplakia, etc. (for a review, see Ref. 15). About 90% of the human population is infected with EBV, and enhanced viral replication and pathology are observed in immunocompromised individuals, e.g., organ transplantees and AIDS patients (16–18). Cross-linking of EBV/CR2 receptors on human platelets causes the aggregation and activation of the latter (11). However, no immunologic consequence of the potential binding of EBV with human platelets has been described to date. We report here that the interaction of EBV with platelets causes the release of TGF-β from the latter. TGF-β is the most immunosuppressive biological agent (3), and its release from platelets after binding of EBV may be an important contributing factor in the immunosuppression observed in acute and chronic EBV infections.

Materials and Methods

Platelet preparations

Platelet preparations were obtained using a modified protocol (19). Briefly, peripheral venous blood was collected from healthy normal donors in citrated blood tubes (Vacutainer, Becton and Dickinson, San Jose, CA) without tourniquet. The first 2 to 3 ml of blood were discarded. The blood was diluted 9:1 in ACD buffer, which is composed of 1.5% citric acid, 2.5% sodium citrate, and 2% dextrose. Platelet-rich plasma was obtained after centrifugation at 150 $\times$ *g* for 15 min.

The platelets were pelleted at 2000 $\times$ *g* for 15 min and washed twice in HEPES buffer containing 10 mM HEPES (7.4), 119 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, and 25 mM $NaHCO_3$. The washes contained 0.5 mg/ml of apyrase (Sigma Chemical Co., St. Louis, MO) and 0.1 U/ml of hirudine (Sigma Chemical Co.). The pelleted platelets were gently resuspended in HEPES buffer, counted in hemocytometer after dilution with 1% ammonium oxalate, and adjusted to a concentration of 1×10^8 platelets/ml. The platelet preparations obtained this way always contained $< 10^4$ white blood cells/ml (data not shown).

Virus preparations

EBV-producing cell lines P3HR1 (20) and B95-8 (21) were used in this study as sources of EBV. Cell-free supernatants were obtained from 1-wk-old cultures of these cell lines after passing through a 0.45-μm pore filter (Nalge Co., NY) as described previously (22). The supernatants were concentrated by centrifuging at 45,000 $\times$ *g* for 90 min at 4°C. The resulting viral pellets were washed and resuspended in ice-cold PBS (pH 7.4). Five hundred-fold concentrations of the supernatants relative to the original culture volume were obtained. The titration of the viral preparation was conducted by using EBV Ag assays on target cell lines, i.e., the induction of Epstsein-Barr virus-encoded nuclear Ags (EBNA) in BJA-B cells for B95-8 and of early Ag (EA) in Raji cells for P3HR1, as described previously (22). The viral stocks contained 2.5×10^5 EBNA-inducing U/ml and 2×10^6 EA-inducing U/ml. For heat inactivation, the virus preparations were heated at 56°C for 30 min. For UV inactivation, the viruses were

Laboratory of Immunovirology, Pediatric Research Center, and Department of Microbiology and Immunology, University of Montreal and Ste-Justine Hospital, Montreal, Quebec, Canada

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[2] Address correspondence and reprint requests to Dr. Ali Ahmad, Laboratory of Immunovirology, Ste-Justine Hospital, 3175 Côte Ste-Catherine, Montreal, Quebec, Canada H3T 1C5. E-mail address: ahmada@justine.umontreal.ca

[3] Abbreviations used in this paper: CR2, receptor for C_3d fragment of complement; EBNA, Epstein-Barr virus-encoded nuclear antigens; EA, early antigen.

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exposed to a UV source for 30 min that delivered 4000 μJ/s. UV-treated and heat-inactivated viruses were noninfectious, since they did not induce any EA or EBNA upon infection of target cells.

Incubation of platelets and preparation of samples for TGF-β assay

Half-milliliter preparations of the platelet suspension were incubated with 50 μl of the virus stocks in 0.5-ml tubes, gently mixed, and incubated at 37°C in 5% CO_2 atmosphere for 1 h unless indicated otherwise. The supernatant was collected after pelleting platelets at 2000 × *g* for 15 min and was stored at −80°C until titrated for TGF-β activity.

Monoclonal Abs

Anti-CR2 mAb OKB7 was obtained from Orthodiagnostics (Mississauga, Canada). TGF-β neutralizing polyclonal Ab was obtained from R&D Systems (Minneapolis, MN). Both Abs were used at a concentration of 10 μg/ml.

Determination of TGF-β

Cells of the mink lung epithelial line Mv1Lu (CCL64, American Type Culture Collection, Rockville, MD) were used as targets for the detection of TGF-β bioactivity in the supernatants obtained as described above. The procedure was previously explained (23). Briefly, CCL64 cells were trypsinized, washed twice with RPMI 1640 containing 1% heat-inactivated FBS and resuspended at a concentration of 10^5 cells/ml in RPMI 1640 containing 2% heat-inactivated FBS. One hundred microliters of this cell suspension was added in triplicate to the wells of a 96-well flat-bottom microculture plate. The culture medium was aspirated the next day, and 200 μl of fresh RPMI 1640 containing 2% heat-inactivated FBS with 50 μl of the supernatant (to be tested for TGF-β activity) was added to the wells. The cultures were pulsed for 6 h with 1 μCi of [^{3}H]thymidine (sp. act., 60 Ci/mmol; ICN Pharmaceuticals, Montreal, Canada). After pulsing, the wells were washed three times with PBS, and cells were lysed with 150 μl of 2% NaOH. The [^{3}H]thymidine incorporation was determined in 100 μl of the cell lysate after adding 4 ml of a liquid scintillation mixture. Before addition to the assay wells, each supernatant was heated at 95°C for 5 min and placed on ice to activate TGF-β.

Statistical analysis

The average counts per minute from triplicate wells between different treatments were compared using Student's *t* test as previously described (24). The differences between means were considered significant at the 5% level of confidence.

Results

Release of TGF-β from platelets after incubation with EBV

The supernatants of platelets incubated with EBV (B95-8 or P3HR1) were processed as described in *Materials and Methods*. These supernatants, when tested in the TGF-β assay, showed significant inhibitory activity ($p \leq 0.05$) on the growth of CCL64 cells. Both EBV strains, P3HR1 and B95-8, showed this activity (Fig. 1*A*). Approximately 50% inhibition of [^{3}H]thymidine incorporation was observed when supernatants from platelets

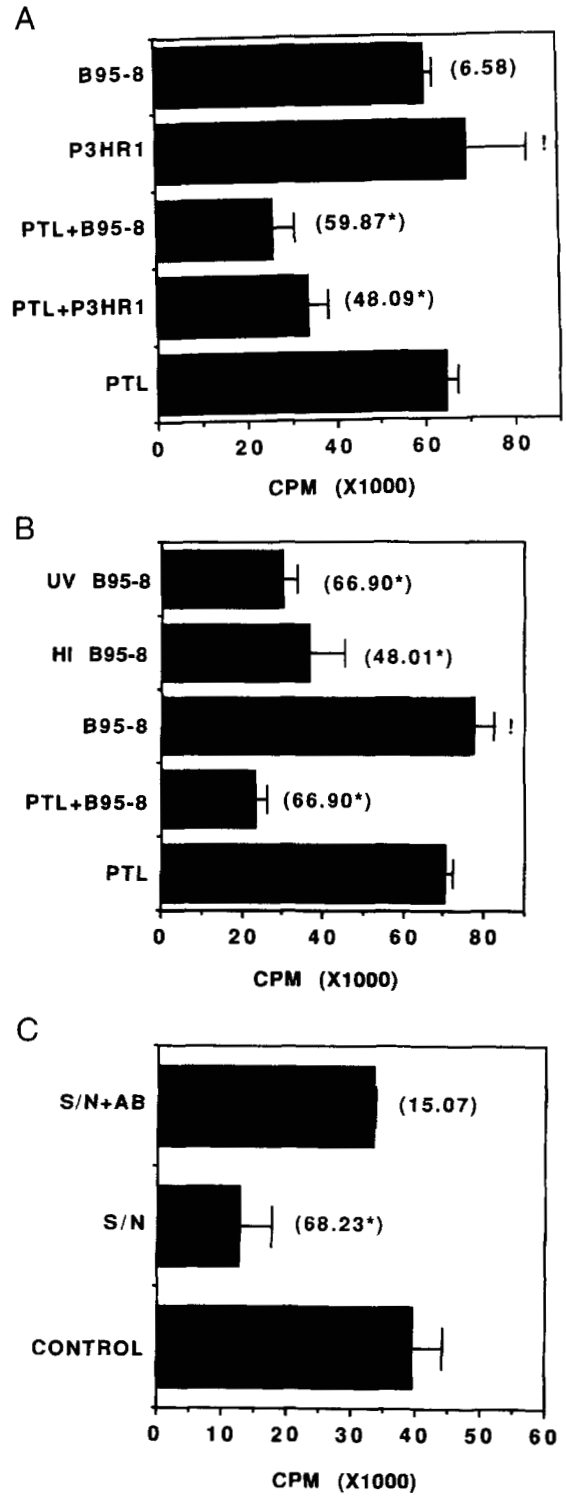

FIGURE 1. Release of TGF-β from platelets upon incubation with EBV. *A*, Platelets were incubated alone or with P3HR1 or B95-8 for 1 h and pelleted by centrifugation. The supernatants (SN) were tested for TGF-β bioactivity by their inhibition of [^{3}H]thymidine incorporation of CCL-64 cells. Shown here are the average ± SD [^{3}H]thymidine incorporation (counts per minute) from three replicate microcultures. The percent inhibition of counts per minute by different SN compared with the counts per minute of CCL-64 microcultures in the presence of SN obtained from platelets alone is given in parentheses on *top* of each bar. The designation PTL in the figure refers to the SN obtained from the platelets incubated alone; PTL+P3HRI refers to the SN from platelets incubated with P3HRI; PTL+B95-8 refers to the SN from platelets incubated with B95-8; and B95-8 and P3HRI refer to the SN obtained from the incubation of the respective virus strain in the HEPES buffer. * Indicates significant ($p \leq 0.05$) inhibition, and ! indicates no inhibition. *B*, Incubation of platelets with heat-inactivated or UV-irradiated EBV showed a significant ($p \leq 0.05$) inhibitory effect. The designations UV B95-8 and HI B95-8 in the figure refer to the SN obtained from the platelets incubated with UV- and heat-inactivated B95-8, respectively; the other designations are the same as above. *C*, The effect of pretreatment of SN obtained from the incubation of platelets and EBV with TGF-β-neutralizing Ab on the growth of CCL-64 cells. The growth inhibitory effect of the SN was significantly ($p \leq 0.05$) abrogated by this treatment. The designation CONTROL in this figure refers to the supernatant obtained from the incubation of platelets in the HEPES buffer alone; S/N refers to the supernatant obtained from the platelets incubated with B95-8; S/N+AB refers to the supernatant obtained from the platelets incubated with B95-8 but treated with anti-TGF-β Ab before addition to the CCL-64 microcultures.

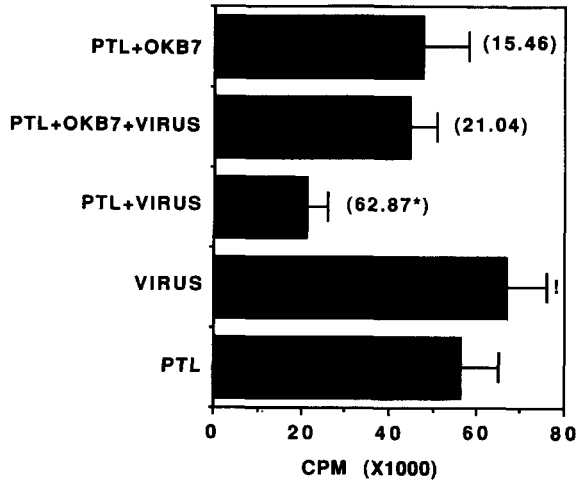

FIGURE 2. The effect of OKB7 on the EBV-mediated release of TGF-β from platelets. Platelets were preincubated for 30 min at room temperature with PBS or OKB7 and then incubated with EBV for 1 h along with controls. Supernatants (SN) from these incubations were tested for growth inhibitory effect of CCL-64 cells. Shown here are the average [^{3}H]thymidine uptake (counts per minute) ± SD from three replicate microcultures. The inhibitory effect (percentage) of various SN is also indicated. The designation PTL in the figure refers to the SN obtained from incubation of platelets alone in the HEPES buffer; VIRUS refers to the SN obtained from the incubation of B95-8 alone in the buffer; PTL+VIRUS refers to the SN from platelets incubated with B95-8; PTL+OKB7+VIRUS refers to the SN obtained from platelets preincubated with OKB7 and then with B95-8; PTL+OKB7 refers to the SN from platelets incubated with OKB7. * indicates significant ($p \leq 0.05$) inhibition, and ! indicates no inhibition. Note that the difference between PTL+OKB7+VIRUS and PTL+VIRUS is also statistically significant ($p \leq 0.05$).

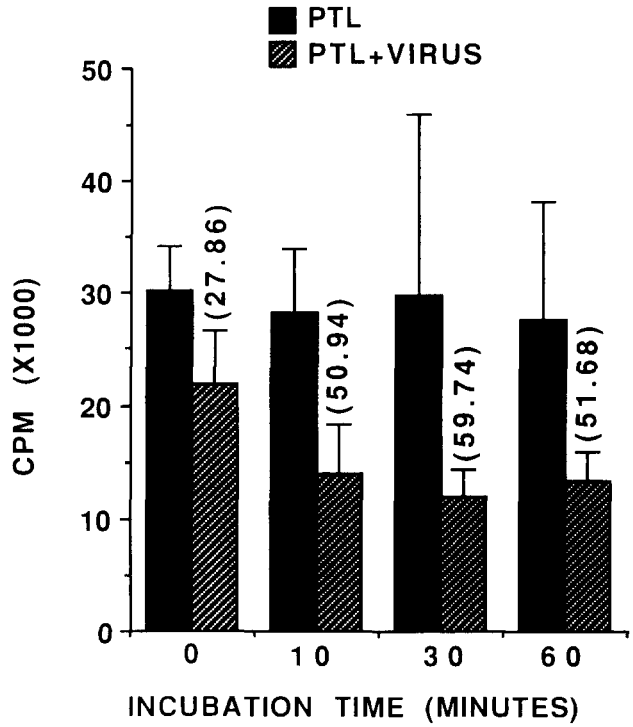

FIGURE 3. Kinetics of TGF-β release from platelets incubated with EBV. Platelets were incubated alone or with EBV for different lengths of time, and their SN were obtained and tested for TGF-β bioactivity on CCL-64 cells. [^{3}H]thymidine uptake (counts per minute) was determined for three replicate microcultures of CCL-64 cells in the presence of SN obtained from platelets alone (PTL) or after incubation with EBV (PTL+VIRUS). The zero time point indicates that the virus was added to the platelets, they were immediately pelleted, and SN was obtained. The percent inhibition is indicated on *top* of each bar.

coincubated with viruses were added to the assay wells. Since in preliminary experiments both B895-8 and P3HR1 EBV strains were found equally effective in inducing the release of TGF-β, only B95-8 virus was used in subsequent experiments. Both UV-treated and heat-inactivated virus preparations, which were non-infectious for permissive target cells, were able to mediate this release (Fig. 1*B*), indicating that the binding of the virus to the platelets was sufficient to cause this release. To determine whether the inhibitory effect of the supernatants obtained from platelets incubated with EBV was specifically due to the presence of TGF-β1, the supernatants were pretreated with TGF-β1-neutralizing Ab for 30 min at room temperature (final concentration of 10 μg/ml) before testing for growth inhibition of CCL64 cells. As shown in Figure 1*C*, this treatment significantly ($p \leq 0.05$) abrogated the inhibitory effect of the supernatant, indicating that the this inhibitory effect was specifically due to the presence of TGF-β1 released from the platelets.

Inhibition of TGF-β release by OKB7 mAb

OKB7 is a murine CR2-specific mAb that has been shown to inhibit the binding of EBV to CR2 on target cells (14, 25). We preincubated platelets with OKB7 to determine whether coating CR2 with this mAb on platelets would inhibit TGF-β release by inhibiting the binding of EBV to platelets. As shown in Figure 2, OKB7 itself did not cause the release of TGF-β from platelets, but significantly inhibited EBV-mediated release when platelets were preincubated with OKB7, indicating that the binding of EBV to CR2 on platelets is the specific event that caused the release of TGF-β and that inhibition of this binding also inhibits this release.

Kinetics and mechanism of TGF-β release

A time-course study was conducted to determine the kinetics of the TGF-β release. For this purpose, platelet suspensions were incubated with EBV for different lengths of time, and the supernatants were obtained and titrated for TGF-β activity. As shown in Figure 3, the TGF-β activity was detected as early as 10 min after incubation with EBV, the earliest time point examined. This activity was still present in the supernatant 4 h after incubation (data not shown). More importantly, platelets that were incubated with the virus for zero time (i.e., platelets that were centrifuged immediately after the addition of EBV to collect supernatants) also showed some TGF-β activity. This TGF-β activity could not be present in the virus preparation, since the incubation of this virus preparation alone in the HEPES buffer (used for platelet incubation) did not show any inhibitory effect on the [^{3}H]thymidine uptake of CCL-64 microcultures (Fig. 1, *A* and *B*). These experiments suggest that TGF-β is released from platelets within minutes after contacting EBV. This quick release also suggests that TGF-β is released from preformed secretory granules of the platelets after virus binding, and prolonged incubation of the virus with platelets does not increase this release. In order to verify that de novo synthesis of TGF-β is not involved in this experimental system, platelets were incubated with EBV for 1 h, then washed and reincubated with EBV for 1 h. The supernatants obtained from these two successive incubations were tested for the presence of TGF-β activity. As shown in Table I, supernatants from the first, but not from the second, incubation showed TGF-β activity, indicating that the platelets had released their entire TGF-β content during the first

Table I. *Lack of TGF-β release from platelets upon second incubation with EBV*[a]

Expt. No.	First Incubation	cpm	Second Incubation	cpm
1	PBS	41,193 ± 6621	B95-8	20,542 ± 5692
2	B95-8	24,645 ± 2514	B95-8	39,410 ± 2380

[a] Platelets were incubated with PBS or B95-8 for 1 h and supernatants (SN) were collected. After this first incubation, these platelets were washed and re-incubated separately with virus for 1 h. The SN from the first and second incubations were tested for TGF-β bioactivity. Shown here are average cpm ± SD of three separate microculture wells of CCL-64 cells to which these SN were added. The SN from platelets from the first incubation with B95-8 showed significant $p \leq 0.01$ inhibitory effect on ^{3}[H]thymidine incorporation while no inhibitory effect was observed in SN obtained from subsequent incubation of these platelets with EBV.

incubation. The platelet suspension incubated twice in similar fashion released TGF-β upon the second incubation only when the first incubation did not contain EBV. Furthermore, attempts to detect TGF-β messages in platelets with and without incubation with EBV using Northern blots and a semiquantitative reverse transcription-PCR assay produced negative results (data not shown; see *Discussion*).

Discussion

Human platelets have been documented to bear CR2, and the cross-linking of these receptors on platelets has been shown to cause aggregation and activation of the latter (11). We provide evidence here that the binding of EBV to its receptors on human platelets causes the release of TGF-β from the latter. TGF-β is the most potent immunosuppressive substance found to date in the human body. It can modulate both cellular and humoral immune responses of the host and may stimulate or inhibit cell proliferation depending upon the target cells and the culture conditions used (3). Although human TGF-β exists in several isoforms that are produced from a wide variety of cells and tissues, platelets represent the richest source of TGF-β1 in the human body (3). TGF-β1 is the prototype member of TGF-β family and the main isoform that mediates TGF-β-like activities in the human body. EBV and, more specifically, its *trans*-activator Zta have been shown to induce the secretion of TGF-β from infected human PBMC (26). However, to our knowledge this is the first report describing the EBV-induced release of this immunosuppressive agent from human platelets, and it is tempting to speculate that this source of TGF-β may, at least in part, be responsible for the immunosuppression that accompanies EBV infections. Increased serum concentrations of TGF-β have also been reported in AIDS patients (27). It is noteworthy that in AIDS patients and other immunocompromised individuals (e.g., organ transplantees) there is enhanced EBV replication, and elevated EBV titers have been found in their blood and saliva (16–18, 28). In these individuals, EBV may potentially induce the release of TGF-β from their platelets. Decreased platelet counts (thrombocytopenia) observed in AIDS patients and acute EBV infections (29, 30) may at least in part be due to the EBV-induced aggregation and secretion of these blood elements, although definitive proof of this is presently lacking.

Our data suggest that binding of EBV to its receptor is the specific event that causes TGF-β release. mAb OKB7, which blocks the binding of EBV to CR2, also blocked this release. Furthermore, pretreatment of EBV with a neutralizing mAb, 72A1, also inhibited this release (data not shown). The CR2 receptor is a 140-kDa type II integral membrane protein that is a member of the regulators of complement activation gene family. The products of this gene family are characterized by the presence of repeating motifs of 60 to 70 amino acids, designated short consensus repeats or complement control modules in their extracellular region. CR2 consists of 15 or 16 short consensus repeats, a transmembrane domain, and a short carboxyl-terminal intracellular domain (31). The short intracellular domain of CR2 argues against its ability to transduce intracellular signals on its own, although this domain may potentially associate with p53 and p68, a calcium binding protein (32, 33). On B cells, however, CR2 exists as a complex with CD19, TAPA-1 (target of anti-proliferation Ab), and CR1 and transduces signals via CD19 and/or TAPA-1 (reviewed in Ref. 34). Thus, CR2 is an important component of the B cell Ag receptor complex, CD19/TAPA-1/CR2, and can augment the immune response to an Ag if the latter is bound to the natural ligand of CR2, i.e., C_3dg (35, 36). CR2 occurs on human B cells, a subpopulation of mature T cells, immature thymocytes, epithelial and follicular dendritic cells, and platelets (11, 37–39). Activation of B cells results in phosphorylation of CR2. Cross-linking of CR2 induces DNA synthesis in B cells pretreated with phorbol esters (25, 40). The interaction of human PBMC with EBV dysregulates the secretion of IL-6, TNF-α, and other cytokines that are blocked by OKB7 mAb as well as by protein kinase C inhibitors (41, 42; our unpublished observations). The ability of EBV to induce the release of TGF-β from platelets is also blocked by OKB7, indicating the similarity of the signaling mechanisms initiated by EBV binding to CR2 on platelets and on PBMCs. Recently, it has been shown that EBV can also modulate the proliferation of double-positive ($CD4^+$ $CD8^+$) immature thymocytes and hence may potentially interfere with the elimination of self-reactive T cells, resulting in autoimmune disorders (43). Our results illustrate yet another example of how EBV can display its enormous immunomodulating potential, i.e., by using an important member of the B cell signaling complex as a receptor.

TGF-β is stored in secretory granules of platelets that contain several other substances, e.g., platelet-derived growth factor, platelet-activating factor, and several plasma proteins, that upon release can cause adhesion and aggregation of platelets. Several platelet agonists can cause platelet activation, secretion, and aggregation (reviewed in Ref. 1). This secretion occurs through an unusual exocytosis (1). Platelets express P-selectin (CD62) on their granule membranes and upon activation translocate it to their surface. Ligands for P-selectin are expressed on the surface of monocytes, neutrophils, NK, and memory T cells. Thus, via P-selectin, activated platelets can interact with these cell types and modulate their activities (10). However, at present it is not known whether EBV binding to platelets selectively causes the release of TGF-β or whether all contents of the secretory granules are exocytosed and EBV-bound platelets exhibit the full spectrum of characteristics of activated platelets.

In conclusion, we have demonstrated here that binding of EBV to CR2 on the human platelets induces the release of TGF-β from the latter; this release appears to be from the platelet secretory granules and can be blocked by occupation of CR2 by mAb OKB7. This underscores the important, but underemphasized, role that platelets may play in the pathogenesis of infectious diseases and host defense against these diseases, aside from their roles in hemostasis and thrombosis.

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