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Rapid selection of cell subpopulation-specific human monoclonal antibodies from a synthetic phage antibody library

(phage antibody display/human antibodies)

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ABSTRACT **Peripheral blood leukocytes incubated with a semisynthetic phage antibody library and fluorochrome-labeled CD3 and CD20 antibodies were used to isolate human single-chain Fv antibodies specific for subsets of blood leukocytes by flow cytometry. Isolated phage antibodies showed exclusive binding to the subpopulation used for selection or displayed additional binding to a restricted population of other cells in the mixture. At least two phage antibodies appeared to display hitherto-unknown staining patterns of B-lineage cells. This approach provides a subtractive procedure to rapidly obtain human antibodies against known and novel surface antigens in their native configuration, expressed on phenotypically defined subpopulations of cells. This approach does not depend on immunization procedures or the necessity to repeatedly construct phage antibody libraries.**

The construction of libraries of fragments of antibody molecules that are expressed on the surface of filamentous bacteriophage and the selection of phage antibodies (ϕAbs) by binding to antigens offers a powerful means of generating new tools for research and clinical applications (1). This technology has been mainly applied to generate ϕAbs specific for purified antigens that are available in sufficient quantities for solid-phase-dependent selection procedures (reviewed in ref. 2). The effectiveness of such ϕAbs in biochemical and functional assays varies; typically, the procedure used to select ϕAbs determines their utility (2–6).

Human monoclonal antibodies that bind to native cell surface structures are expected to have broad application in therapeutic and diagnostic procedures (7). An important extension of ϕAb display technology would be a strategy for the direct selection of specific antibodies against antigens expressed on the surface of subpopulations of cells present in a heterogeneous mixture. Ideally, such antibodies would be derived from a single highly diverse library containing "every" conceivable antibody specificity. We have recently constructed a library from 49 human germline immunoglobulin heavy-chain variable (V_H) genes fused to a heavy-chain joining segment 4 (J_H4) gene and partly randomized complementarity-determining region 3 (CDR3) sequences varying in length between 6 and 15 amino acids. The CDR3s were designed to contain short stretches of fully randomized amino acid residues flanked by regions of limited variability. Residues in the latter portion of CDR3 were selected on the basis of their frequent occurrence in CDRs of natural antibody molecules (8–10). We reasoned that a designed, semirandom CDR3 would result in an increased frequency of clones producing functional antigen binding sites and, in addition, in a more efficient use of the restricted sequence space within phage display libraries. The synthetic V_H segments were combined with seven different light-chain variable (V_L) genes and expressed as gene III–single-chain Fv (scFv) fragments on the surface of phage, resulting in a library of 3.6×10^8 clones. From this library we isolated monoclonal ϕAbs (mϕAbs) to a variety of different structures (haptens, proteins, and polysaccharides) by selection on solid-phase bound antigen. These phage antibodies proved to be useful reagents in a variety of biochemical assays (6).

In the present experiments we have exploited the combination of the antibody library and flow cytometry to isolate mϕAbs specific for subpopulations of cells present in a heterogeneous mixture. We demonstrate that these antibodies detect known and novel structures on various populations of blood and fetal bone marrow cells.

MATERIALS AND METHODS

Selection of ϕAbs by Cell Sorting. Venous blood was diluted 1:10 in 0.8% NH_4Cl/0.08% $NaHCO_3$/0.08% EDTA, pH 6.8, to remove erythrocytes, and the nucleated cells were pelleted and washed once in phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA). Approximately 10^{13} phage particles bearing antibodies were blocked for 15 min in 4 ml of PBS/4% milk powder. Leukocytes (5×10^6) were added to the blocked phages and the mixture was slowly rotated overnight at 4°C. The following day, cells were washed twice in 50 ml of ice-cold PBS/1% BSA. The pelleted cells were suspended in 50 μl of peridinin chlorophyll protein (PerCP)-conjugated CD3 antibody solution and 50 μl of fluorescein isothiocyanate (FITC)-conjugated CD20 antibody solution. After a 20-min incubation on ice, cells were washed once with PBS/1% BSA and suspended in 500 μl of ice-cold PBS/1% BSA. Cell sorting was performed on a FACSvantage fluorescence-activated cell sorter (Becton Dickinson). For each subpopulation, 10^4 cells were sorted in 100 μl of PBS.

Propagation of Selected Phages. Phages were eluted from the cells by the addition of 150 μl of 76 mM citric acid (pH 2.5) in PBS followed by incubation for 5 min at room temperature. The mixture was neutralized with 200 μl of 1 M Tris·HCl, pH 7.4. Eluted phages were used to infect *Escherichia coli* XL1-Blue (Stratagene) and the bacteria were plated on TYE medium (11) containing the appropriate antibiotics and glucose. Bacterial colonies were counted, scraped from the plates, and used as an inoculum for the next round of phage rescue (11).

Preparation of mϕAbs and scFv Fragments and Immunofluorescence Analysis. Phages were prepared from individual ampicillin-resistant colonies grown in 25 ml of 2TY medium (11), purified by polyethylene glycol (PEG) precipitation, resuspended in 2 ml of PBS, filtered (0.45 μm), and stored at

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Abbreviations: ϕAb, phage antibody; mϕAb, monoclonal ϕAb; APC, allophycocyanin; BSA, bovine serum albumin; CDR, complementarity-determining region; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; scFv, single-chain Fv; sIgM, surface IgM.

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4°C until further use. scFv fragments were produced in the *E. coli* nonsuppressor strain SF110, which is deficient in the proteases DegP and OmpT (12). In our experience, the stability of scFv produced in SF110 is superior to that of scFv produced in *E. coli* HB2151, which is commonly used for this purpose.

For staining of leukocytes, 100 μl of mϕAb was blocked by adding 50 μl of PBS/4% milk powder for 15 min at room temperature. Leukocytes (5×10^5) in 50 μl of PBS/1% BSA were added and incubated on ice for 1 hr. The cells were washed twice in ice-cold PBS/1% BSA. To detect cell-bound phages, the cells were incubated in 10 μl of a 1:200 dilution of sheep anti-M13 polyclonal antibody (Pharmacia) for 20 min on ice, washed twice, and incubated in 10 μl of a phycoerythrin-labeled donkey anti-sheep polyclonal antibody solution (20 μg/ml) (Jackson ImmunoResearch) for 20 min on ice. The cells were washed and incubated in 10 μl of FITC-labeled CD3 monoclonal antibody and 10 μl of PerCP-labeled CD20 monoclonal antibody. When cells were stained with purified scFv fragments, second- and third-step reagents consisted of the anti-Myc tag-specific antibody 9E10 and FITC- or PE-labeled goat anti-mouse antibodies. After a single final wash, the cells were resuspended in 0.5 ml of PBS/1% BSA and analyzed by flow cytometry.

Fetal bone marrows were from aborted human fetuses (16–22 weeks of gestation) and used according to the guidelines of the institutional review board of Stanford Medical School Center on the use of Human Subjects in Medical Research. Bone marrow cells were obtained by flushing intramedullary cavities of the femurs with RPMI 1640 medium. Pelleted cells were treated with the hypotonic NH_4Cl solution to remove erythrocytes. Fetal bone marrow cells (10^6) were stained with mϕAbs T1, B9, and B28 in combination with a panel of fluorochrome-labeled antibodies including PerCP-labeled anti-Leu4b (CD3), FITC-labeled anti-Leu3 (CD4), allophycoryanin (APC)-labeled anti-Leu2a (CD8), and FITC-labeled anti-CALLA (CD10) monoclonal antibodies (all from Becton Dickinson Immunocytometry Systems) and FITC-conjugated goat anti-human μ, δ, and κ chain-specific polyclonal antibodies (Southern Biotechnology Associates).

RESULTS AND DISCUSSION

After erythrocyte lysis, 5×10^6 peripheral blood cells from a healthy individual were incubated with the phage library and subsequently stained with PerCP-labeled CD3 and FITC-labeled CD20 monoclonal antibodies. Fig. 1*A* shows the flow cytometric identification of B lymphocytes ($CD20^+CD3^-$, black crosses), T lymphocytes ($CD20^-/CD3^+$, gray crosses), eosinophils (high autofluorescence, small black dots), and all other cells (depicted in gray). Ten thousand cells of each population were sorted and the phages bound to the isolated cells were eluted from the cell surface. We reasoned that phages interacting with structures present on all or a majority of cells in the heterogeneous population would be absorbed out by the nonselected cells, effectively resulting in the enrichment of phages specific for the target cells. Further, we anticipated that the considerable shear forces exerted on the cells and attached phage during fluorescence-activated cell sorting would result in selection for relatively high-affinity antibodies. The number of clones obtained after the first round of selection varied between 320 and 1704; note that number of phage clones obtained correlated inversely with the frequency of the cell population in the blood sample (Table 1). The second round of selection resulted in a modest increase in the number of phages eluted from the cells in most but not all cases (Table 1). Similar results were obtained in a second experiment using blood leukocytes from an unrelated donor (results not shown). The low number of phages recovered from cells expressing thousands of potential epitopes may be attributed to a number of factors, including a low, diffusion-dependent on-rate of phage binding to the cells and the stringent sorting-dependent selection procedure.

The phages eluted from the sorted cells were expanded as individual libraries and used in a second round of selection employing the same procedure. Finally, mϕAbs were prepared from individual colonies obtained after the second round of selection. The binding properties of 15 mϕAbs from each sorted population were analyzed by incubation with peripheral blood leukocytes followed by incubation with PE-labeled anti-phage secondary antibody and FITC-labeled CD20 and PerCP-labeled CD3 antibody. After two rounds of selection, between 63% and 100% of the mϕAbs were found to display binding activity leukocytes (Table 1). Fig. 1 illustrates the staining profiles of a negative control mϕAb (*B*); a mϕAb derived from sorting all leukocytes, A1 (*C*); two eosinophil-derived mϕAbs, E1 and E2 (*D* and *E*); two T-cell-derived mϕAb, T1 and T2 (*F* and *G*); and two B-cell-derived mϕAbs, B9 and B28 (*H* and *I*). The positions of monocytes and neutrophils are indicated in Fig. 1*B* with M and N, respectively. scFv fragments were produced from each mϕAb clone. For all clones, identical results were obtained for whole phage antibodies and isolated scFv fragments, albeit some loss of signal intensity was observed when the latter were used (e.g., compare Figs. 1*G* and 2*C*). The 15 mϕAbs selected on all leukocytes showed identical staining patterns: all granulocytes, eosinophils, and monocytes stained homogeneously bright. All the T lymphocytes stained, but with varying intensity. Strikingly, no binding to B lymphocytes was observed (Fig. 1*C*). Among the 15 mϕAbs selected for binding to eosinophils, 2 staining patterns were discernible. Both mϕAbs bound to all eosinophils and monocytes; the staining profile of granulocytes differed between the two mϕAbs. mϕAb E2 reacted with the majority of T cells, whereas virtually no staining of T cells was observed with E1. Conversely, E2 did not bind to B cells, whereas E1 stained virtually all B cells (compare Fig. 1 *D* and *E*). Two staining patterns could be distinguished among the 12 mϕAbs selected for binding to T lymphocytes. mϕAb T2 dimly stained a subpopulation of B cells, T cells, and granulocytes but not monocytes and eosinophils (Fig. 1*F*). T1 exclusively and brightly stained a subpopulation of T lymphocytes comprising ≈50% of $CD3^+$ cells. Finally, among mϕAbs selected from B cells, three staining patterns were distinguishable: ≈50% of the peripheral blood B cells stained with mϕAb B9 (Fig. 1*H*), B28 stained all $CD20^+$ peripheral blood B cells (Fig. 1*I*), and B11 stained virtually all leukocytes (data not shown).

mϕAbs T1, B9, and B28 were selected for further characterization. In four-color staining experiments with CD3, CD4, CD8, and T1 antibodies, T1 was shown to bind to $CD8^+$ cells and not to $CD4^+$ cells (Fig. 2 *A–C*). Immunofluorescent staining of COS cells transiently transfected with cDNAs encoding the CD8 α chain, the CD8 β chain, or both demonstrated that T1 recognized cells expressing the CD8 αα homodimer (results not shown). We conclude that T1 recognizes an epitope on the CD8 α chain.

In costaining experiments with a panel of antibodies defining various cell subpopulations, B9 did not stain $CD2^+$, $CD56^+$, $CD14^+$, or $CD15^+$ populations of cells, consistent with a B-cell-specific expression pattern (results not shown). Triple staining of B9 with CD20 and antisera specific for the immunoglobulin μ, δ, γ, α, ε, κ, and λ chains revealed that B9 marker expression did not concur with any of the immunoglobulin isotypes (e.g., see Fig. 2 *D–F*). Triple staining of purified tonsillar B cells with mϕAb B9 or B28, CD19, and CD10 or μ heavy-chain-specific antibodies confirmed that B28 bound to all and B9 bound to a subpopulation of $CD19^+$ tonsillar B cells (data not shown). Germinal-center B cells ($CD19^+CD10^+$) uniformly lacked the antigen recognized by B9 (Fig. 2*G*). In human bone marrow, the CD19 marker is expressed from the earliest pro-B cell to the virgin, $sIgM^+$ B-cell stage. Triple

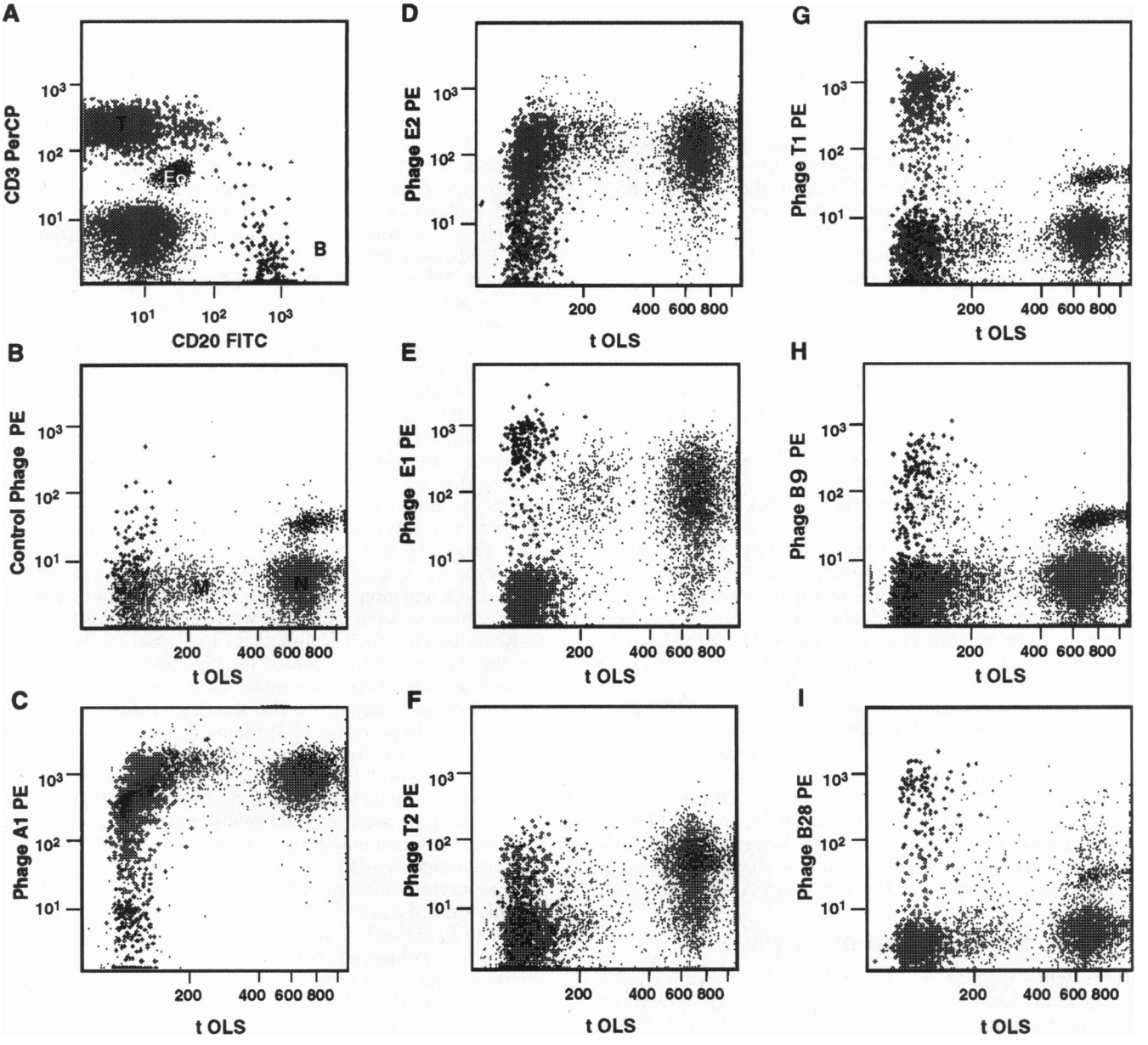

FIG. 1. Staining of blood leukocytes with mϕAbs and analysis by flow cytometry. The subpopulations used for sorting are depicted in *A*. T; T lymphocytes; B, B lymphocytes; Eo, eosinophils. The following notation was used to identify the individual subpopulations in all panels: CD20 B lymphocytes ($CD20^+CD3^-$, black crosses), T lymphocytes ($CD20^-CD3^+$, gray crosses); eosinophils (high autofluorescence, small black dots; all other cells (depicted grey). The list-mode data were analyzed with custom flow-cytometry presentation software made by B. Verwer at Becton Dickinson Immunocytometry Systems. tOLS, transformed orthogonal light scatter.

staining of fetal bone marrow cells with CD19, sIgM, and B9 or B28 antibodies demonstrated that B9 and B28 antigens were not expressed during B-lineage differentiation (Fig. 2 *H* and *I*). We conclude that the structures detected by mϕAbs B9 and B28 are expressed at a very late stage of B-cell development, presumably after newly generated $sIgM^+$ B cells have left the bone marrow. To our knowledge, B-cell-specific markers with such expression patterns have not been described previously.

Table 1. Number and staining profiles of mϕAbs eluted from two rounds of selection on subpopulations of periferal blood leukocytes

Sorted population	No. of mϕAbs		No. of positive clones	No. of staining profiles
	Round 1	Round 2		
"All" leukocytes	640	980	15/15	1
Eosinophils	1280	390	11/15	2
T cells ($CD3^+$)	320	3330	15/15	2
B cells ($CD20^+$)	1704	6000	10/16	3

Nucleotide sequence analysis was used to establish V_H and V_L gene utilization and heavy-chain CDR3 composition of the scFv antibodies obtained from the sorted subpopulations (Table 2). ScFv derived from different clones with the same staining profile showed identical nucleotide sequences of CDR3s. The mϕAbs with different staining patterns were encoded by various combinations of V_H and V_L chains, with an overrepresentation of the DP32 gene fragment, and comprised CDR3 loops varying in length between 6 and 12 amino acids.

We have described a fluorescence-activated cell-sorting method for isolating human ϕAbs against subpopulations of cells in a heterogeneous mixture. The nonselected cells in the mixture appear to absorb out phages that bind to structures shared by the target and absorber cells, in effect providing a subtraction at the protein level. The procedure is independent

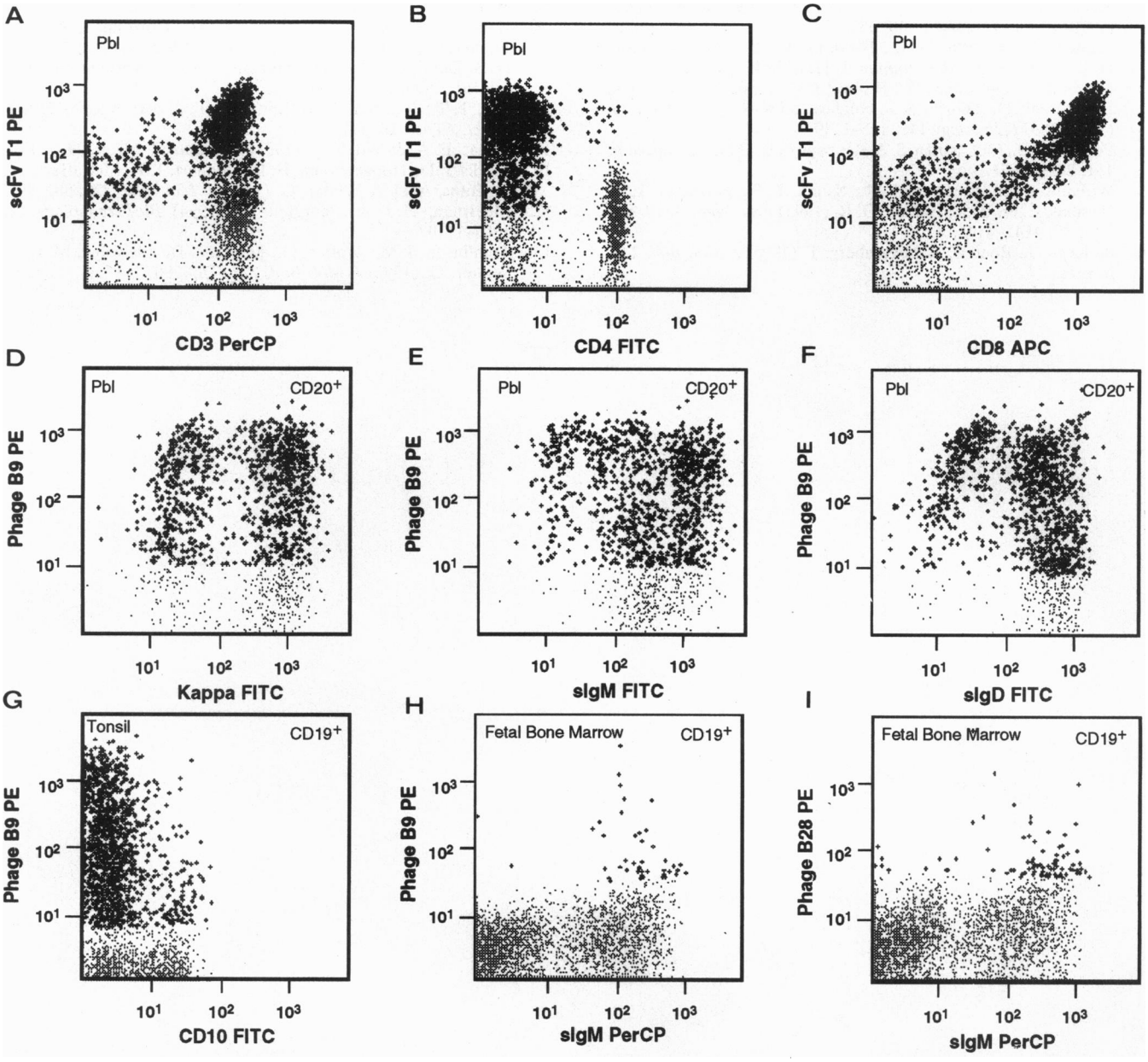

FIG. 2. Characterization of mϕAbs T1, B9, and B28. Unseparated peripheral blood leukocytes (Pbl), purified tonsillar B cells, and unseparated fetal bone marrow cells were stained with T1, B9, and B28 in combination with a panel of fluorochrome-labeled monoclonal antibodies including PerCP-labeled CD3, FITC-labeled CD4, APC-labeled CD8, FITC-labeled CD10, and FITC-labeled μ, δ, and κ chain-specific polyclonal antibodies. In *D–F*, only the $CD20^+$ cells are shown. In *G–I*, only $CD19^+$ cells are shown. Black and gray dots: cells staining positive and lacking staining with a mϕAb, respectively. sIgM, surface IgM.

of immunogenicity of target structures, since the antibodies are isolated from a single large semisynthetic phage antibody display library, constructed entirely *in vitro*. These features render this technology particularly useful for the isolation of antibodies against very rare populations of cells such as hematopoietic stem cells in bone marrow and antibodies against nonimmunogenic structures, including putative tumor-specific antigens present on malignant cells. The antibodies are directed against cell-bound proteins in their native configuration and, due to the stringent selection procedure, are presumably of high enough affinity to be applicable in flow cytometric analysis and as reagents for immunodiagnostics and immunotherapy, such as tumor imaging and bispecific antibody therapy. Finally, the antibody fragments in our library are built from human V_H and V_L segments and contain CDR3s designed to resemble the CDR3s found in natural antibodies, perhaps resulting in reduced immunogenicity when applied in immunotherapy as scFv fragments or whole antibodies.

Table 2. V_H and V_L gene utilization and deduced amino acid sequence of CDR3s of selected mϕAbs

mϕAb	CDR3*	V_H	V_L
A1	RMRFPSY	DP32	Vλ3
E1	RLRSPPL	DP32	Vλ2
E2	RAWYTDSFDY	DP45	Vκ1
T1	KWLPPNFFDY	DP32	Vκ3
T2	RSTLADYFDY	DP69	Vλ3
B9	KGVSLRAFDY	DP31	Vκ1
B28	RGFLRFASSWFDY	DP32	Vλ3

Nomenclature of the V_H genes is as in ref. 13.
*Amino acid residue 94 is underlined (8).

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