

## MONOCLONAL ANTIBODIES REACTIVE WITH THE MOUSE INTERLEUKIN 5 RECEPTOR

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IL-5 is a glycoprotein secreted by activated T lymphocytes (1, 2). cDNAs encoding both human and mouse IL-5 were obtained (3, 4). Purified natural or recombinant IL-5 have been found to possess various biological activities on different cell types. IL-5 induces the expression of receptors for IL-2 (5, 6) and promotes both proliferation and maturation of mouse B lymphocytes into antibody-secreting cells (7). Together with IL-2, IL-5 supports the expression of cytotoxic functions by activated thymocytes (2). Also, it acts as differentiation factor for cells of the eosinophil lineage from man and mice (8, 9). The biologically active form of mouse IL-5 is an  $M_r$  40,000–50,000 dimer (2, 10). IL-5 binding sites displaying high ( $K_d$   $10^{-11}$  M) and low ( $10^{-9}$  M) affinity have been found on both BCL1 tumor cells and the IL-5-dependent B<sub>13</sub> cell line (2, 10). Crosslinking [<sup>125</sup>I]IL-5 to these cells has revealed an  $M_r$  45,000–50,000 membrane component as candidate for the IL-5-R or at least one of its components (2, 10). Here we describe two rat mAbs, called R52.120 and R52.625, that recognize epitopes on the IL-5-R very close or identical to the binding sites for IL-5.

### Materials and Methods

*Animals.* BALB/c, DBA/2, C57BL/6, and C57BL/6 *nu/nu* mice and Lewis rats were obtained from the Institute für Biologisch Medizinische Forschung AG, Füllinsdorf, Switzerland.

*Cell Lines.* The continuous proliferating cell lines CTLL (11), C4-77 (12), and FTH5 (13) were used to assay for IL-2, IL-3, and IL-4 activities, respectively. The B cell lineage cell lines A1, B4, B5, B<sub>13</sub>, C2, C5, D3, IA, IB, IC, and ID were obtained from cultures in which the LyH7 or LyD9 PRO-B lymphocyte clones (14) were induced to differentiate in vitro with bone marrow stroma cells or splenic nonlymphoid accessory cells. These cell lines represent intermediate stages of differentiation between the PRO-B parental clones and mature IgM<sup>+</sup> B lymphocytes, and they express functional receptors for both IL-3 and IL-5 (R. Palacios, unpublished observations).

*Interleukins and Assays for Interleukin Activity.* Culture supernatants of X63-Ag-653 myeloma cells transfected with the bovine papilloma virus-based expression vector BMGneo containing cDNAs encoding mouse IL-2, -3, -4, and -5, respectively, were used as sources of interleukins (15).

IL-2, IL-3, and IL-4 were tested for their capacity of supporting growth of IL-2-dependent CTLL ( $10^4$  cells/well), IL-3-dependent C4-77 ( $10^4$  cells/well), and IL-4-dependent FTH5 ( $10^4$  cells/well) cells as described in detail elsewhere (12–14). IL-5 activity was tested by its capacity to support growth of the IL-5-sensitive B<sub>13</sub> cell line. B<sub>13</sub> cells were plated at a density of  $5 \times 10^4$  cells/ml in 200  $\mu$ l culture medium (DMEM supplemented with 10% FCS, antibiotics,

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and 50  $\mu$ M 2-ME) in flat-bottomed microtiter plates in the presence of different concentrations of IL-5 and were incubated for 36 h at 37°C. Cell proliferation was determined by [<sup>3</sup>H]thymidine uptake (0.5  $\mu$ Ci/well, sp act 5 mCi/mmol; Radiochemical Centre, Amersham, UK) during the last 12 h of the culture period. 1 U of IL-5 was defined as the amount of IL-5 required for half-maximal proliferation of the B<sub>13</sub> cells. IL-5 was also tested for its capacity of promoting maturation of purified small resting C57BL/6 *nu/nu* splenic B cells to IgM-secreting cells. Small resting splenic B cells were purified by velocity sedimentation as originally described by Miller and Phillips (16). These cells were then cultured at  $5 \times 10^5$  cells/ml in the presence of different amounts of IL-5. The number of IgM-secreting cells was determined at day 5 of culture with the use of the protein A plaque assay (17).

**Production of mAbs.** Lewis rats were immunized on day 0 with  $2 \times 10^7$  B<sub>13</sub> cells emulsified in CFA in the hind foot path. On day 4 and 7 the rats were boosted with  $2 \times 10^7$  B<sub>13</sub> cells in PBS in the same foot path. On day 8 the regional lymph nodes were removed and the cells were fused to the azaguanine-resistant nonsecreting hybridoma cell line SP2/0.  $2 \times 10^8$  fused lymph node cells were plated in 40 microtiter plates in the presence of rat thymocytes ( $3 \times 10^6$ /ml) used as filler cells. Culture wells showing positive growth were tested for the presence of antibodies able to block the IL-5-driven proliferation growth of the B<sub>13</sub> cells as follows: B<sub>13</sub> cells were cultured in the presence of 5–10 U of murine IL-5 and 10% hybridoma culture supernatant for 36 h. Proliferation of the cells was measured as described above.

For purification of the mAbs, hybridomas R52.120, R52.625, and M41 (18) were cultured for 48–72 h in serum-free Iscove's (19) medium containing only transferrin. Antibodies were purified from these supernatants by ion exchange over DEAE-cellulose followed by FPLC on superose-12 (Pharmacia Fine Chemicals, Piscataway, NJ).

**IL-5 Binding Assays.** IL-5 was obtained from culture supernatants of X63-Ag-653 myeloma cells transfected with the expression vector BMG neo containing cDNA for mouse IL-5 (15), was purified on an anti-IL-5 column (2) (a gift of Dr. K. Takatsu, Kumamoto University, Kumamoto, Japan), and labeled with <sup>125</sup>I using the Bolton and Hunter reagent ( $\sim 4,000$  Ci/mmol; Radiochemical Centre). IL-5 binding assays were performed as described in detail (10). In short, B<sub>13</sub> cells were incubated with different amounts <sup>125</sup>I-IL-5 for 45 min at 37°C. A 100-fold excess of unlabeled IL-5 were used to determine nonspecific bindings of <sup>125</sup>I-IL-5.

**Immunofluorescence Staining and Flow Fluorometry.** For immunofluorescence studies we routinely used purified mAbs coupled to biotin. Staining of the different cell types was carried out by incubating the cells with 1–5  $\mu$ g of biotinylated antibody for 30 min at 4°C. The cells were washed once and resuspended in buffer (PBS plus 5% FCS and 0.1% NaN<sub>3</sub>) containing an appropriate dilution of FITC-labeled Streptavidin (Amersham). Negative controls were cells incubated with FITC-conjugated Streptavidin only. The cells were washed twice in buffer and a third time in buffer containing propidium iodine to exclude dead cells from analysis.

Fluorescence intensity was measured with a FACScan instrument (Becton Dickinson & Co., Mountain View, CA). A minimum of  $10^4$  cells were analyzed per sample. For cell sorting, essentially the same staining protocol was used, with the exception that NaN<sub>3</sub> and propidium iodine were omitted. Fluorescence-positive cells were sorted using a FACS 440 (Becton Dickinson & Co.) equipped with an argon laser tuned to 488 nm, operating at 200 mW power, and the gates set by forward- and side-scatter on the "lymphoid" population of spleen cells.

**Immunoprecipitation.** B<sub>13</sub> cells were cultured for 6–8 h at a density of  $5 \times 10^6$  cells/ml in methionine-free RPMI medium (Gibco Laboratories, Grand Island, NY) containing 50  $\mu$ M 2-ME, antibiotics, 20 U/ml IL-5, 10% dialyzed FCS, and 100 mCi <sup>35</sup>S-methionine/ml (sp act  $>800$  Ci/mmol, Amersham). Cells were washed three times in PBS and then lysed in a buffer containing 20 mM Tris-HCl, pH 8.6, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 5 mM EDTA, 10 mM NaN<sub>3</sub>, 2% NP-40, and 1 mM PMSF. The cell lysates were processed according to a solid phase immunoprecipitation technique described by Tamura et al. (20). Immunoprecipitates were analyzed by SDS-PAGE (21) followed by autoradiography using X-ray films (Kodak-X-Omat).

## Results and Discussion

From the fusion of regional lymph node cells of two rats immunized with the IL-5-R<sup>+</sup> B<sub>13</sub> cell line, 1,750 hybridomas were obtained. Two out of the 1,750 hybrid-

omas, called R52.120 and R52.625, secreted antibodies that consistently and strongly inhibited the proliferation of B<sub>13</sub> cells driven by IL-5. Both R52.120 and R52.625 were cloned and subcloned by limiting dilution and both secrete a rat IgG1/ $\kappa$  mAb.

Purified R52.120 and R52.625 mAbs inhibited the action of IL-5, so that 1  $\mu$ g/ml of either antibody suppressed by 60–70% the proliferation of B<sub>13</sub> cells promoted by 5 U of IL-5 (Fig. 1 D). Neither antibody, even at a concentration as high as 20  $\mu$ g/ml, had any effect on the proliferation driven by as little as 1 U of IL-2 or IL-4, on their respective target cells (Fig. 1, A and C). Similarly, the mAbs did not inhibit the IL-3-induced proliferation of three IL-3-reactive cell lines (illustrated with one example in Fig. 1 C). A special case is the B<sub>13</sub> cell line, used to produce the IL-5-R-specific mAbs R52.120 and R52.625, which respond to both IL-3 and IL-5. High concentrations (20  $\mu$ g/ml) of both mAbs inhibited by 50% the proliferative response to IL-3. The inhibitory effect of the antibodies on the IL-3-driven response of B<sub>13</sub> cells but not of the other IL-3-sensitive cell lines might indicate an interesting connection of the IL-3 and IL-5 responsiveness and, therefore, of the corresponding receptors on B<sub>13</sub> cells.

Next we tested R52.120 and R52.625 mAbs for their capacity of inhibiting binding of radiolabeled IL-5 to B<sub>13</sub> cells. The target cells incubated with [<sup>125</sup>I]IL-5 and a 100-fold excess of unlabeled IL-5 was used to determine nonspecific binding of [<sup>125</sup>I]IL-5; the isotype-matched rat anti-mouse IgM mAb M41 (16) was used as negative control antibody in these experiments. The results (Fig. 2) show that both R52.120 and R52.625 mAbs blocked the specific binding of radiolabeled IL-5 to B<sub>13</sub> cells. 1  $\mu$ g/ml of either antibody inhibited by >40% the binding of IL-5 to B<sub>13</sub> cells. No inhibition of the IL-5 binding to these cells was observed with the irrelevant isotype-matched control antibody (Fig. 2). Moreover, neither R52.120 nor R52.625 mAbs blocked the binding of [<sup>125</sup>I]transferrin to the same B<sub>13</sub> cells. We conclude that the mAbs R52.120 and R52.625 react against the IL-5-R and that the epitopes recognized by them in the receptors must be very close or identical to the IL-5 binding sites.

The next set of experiments was performed to study by immunofluorescence staining

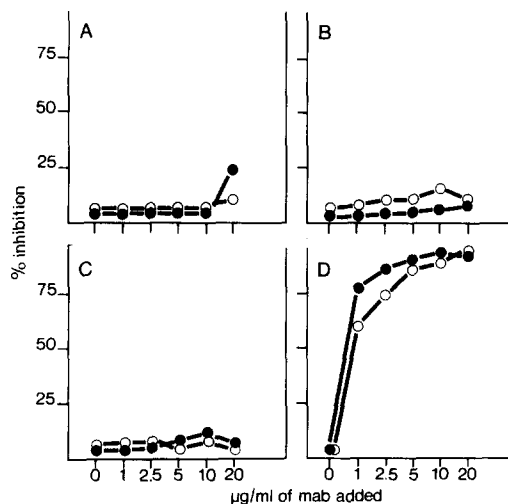


FIGURE 1. mAbs R52.120 and R52.625 inhibit the action of IL-5 but not the action of IL-2, IL-3, or IL-4. Different concentrations of purified R52.120 (●) and R52.625 (○) were added to the cultures set up for assessing their capacity of inhibiting the action of IL-2, IL-3, IL-4, and IL-5 in the following cell responses. (A) Proliferation of CTLL cells driven by rIL-2 (3 U/ml); (B) proliferation of C4-77 cells supported by rIL-3 (5 U/ml); (C) proliferation of FTH5 cells promoted by rIL-4 (5 U/ml); and (D) proliferation of B<sub>13</sub> cells driven by rIL-5 (25 U/ml). In the experiments shown above, the [<sup>3</sup>H]thymidine uptake of target cells cultured in their respective growth factors was as follows: CTLL, 11,600 cpm; C4-77, 12,400 cpm; FTH5, 14,300 cpm; and B<sub>13</sub>, 21,000 cpm. The [<sup>3</sup>H]thymidine uptake of the different target cells cultured in medium without growth factors was as follows: CTLL, 220 cpm; C4-77, 160 cpm; FTH5, 190 cpm; and B<sub>13</sub>, 250 cpm.

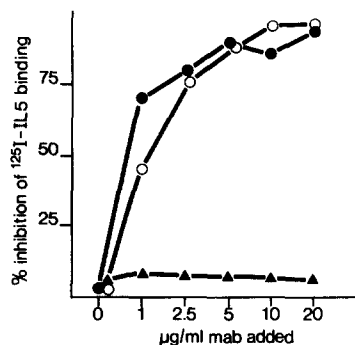


FIGURE 2. R52.120 and R52.625 inhibit the binding of  $^{125}\text{I}$ -IL-5 to  $\text{B}_{13}$  cells.  $\text{B}_{13}$  cells were incubated for 30 min at  $37^\circ\text{C}$  with 40,000 cpm  $^{125}\text{I}$ -IL-5 (35 U) in the presence of different concentrations of purified R52.120 (●), R52.625 (○), and M41 (△). The amount of radioactivity (cpm) bound in the absence of antibodies was 2,600 and in the presence of 100-fold excess of nonlabeled IL-5 was 240.

and flow fluorocytometry (FACS) the binding of both R52.120 and R52.625 mAbs to our collection of nontransformed IL-5-sensitive B cell lineage cell lines, as well as the presence of positive cells among nucleated cells freshly isolated from several hematolymphoid tissues from normal BALB/c, DBA/2, and C57BL/6 mice. Both R52.120 and R52.625 mAbs bound to all 12 B cell lineage cell lines known to grow in IL-5, as illustrated in Fig. 3, *A* and *B* with the data obtained with two such cell lines. Between 2% and 4% spleen cells from all three strains studied were positive for both R52.120 and R52.625 mAbs, but no positive cells could be detected in the adult thymus and lymph nodes (Fig. 3 *C* and data not shown). About 5% of LPS-activated splenic B cells bound R52.120 and R52.625 mAbs, while no binding was observed to Con A-activated splenic T cells. Bone marrow nucleated cells gated for lymphoid cells (by forward- and side-scatter) contained ~5% positive cells for both antibodies (Fig. 3 *D*), and marrow cells gated for myeloid/granulocytes (also by forward-

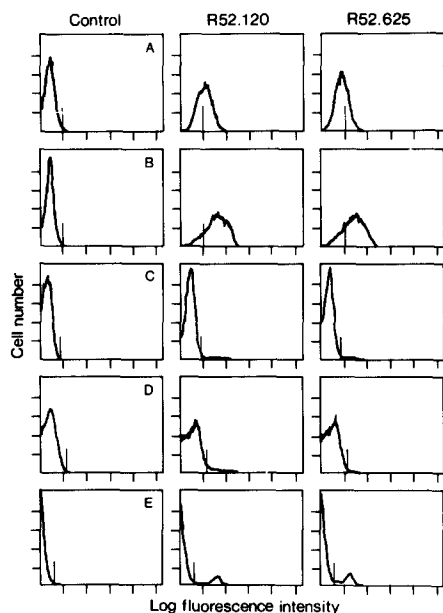


FIGURE 3. Binding of R52.120 and R52.625 mAbs using immunofluorescence staining and flow fluorocytometry to the following cell lines or freshly isolated cell populations. (*A*)  $\text{B}_{13}$  cells, (*B*) LyH7 cells, (*C*) BALB/c spleen cells, (*D*) BALB/c bone marrow cells gated by forward- and side-scatter for the "lymphoid" population, and (*E*) BALB/c bone marrow cells gated by forward- and side-scatter for the "myeloid/granulocyte" population.

and side-scatter) had 10–15% positive cells for both R52.120 and R52.625 mAbs (Fig. 3 E). Peritoneal cells were also found to contain ~10–14% cells that bind both antibodies.

Previous studies have shown that IL-5 promotes maturation of B lymphocytes into antibody-secreting cells (PFC) (7). In light of the finding that both R52.120 and R52.625 mAbs consistently bound to 2–4% lymphoid splenocytes, we wished to know whether among these cells would be B cells responsive to IL-5. Thus, R52.120<sup>+</sup> lymphoid splenocytes were isolated by cell sorter and tested for their capacity to mature into antibody-secreting cells (PFC) in the presence of IL-5. Table I summarizes the results of four experiments. It shows that the frequency of IL-5-inducible PFC increased by a factor of 15–20 in the R52.120<sup>+</sup> cell sorter-purified population as compared with that observed in the unsorted population (Table I). In fact, the frequency of PFC in purified R52.120<sup>+</sup> spleen cells stimulated by IL-5 was similar to that obtained with these cells in the cultures stimulated by LPS (Table I). We conclude that among the R52.120<sup>+</sup> spleen cells, there are B lymphocytes able to mature into antibody-producing cells upon exposure to IL-5.

Yet another piece of experimental evidence for the existence of IL-5-reactive B lymphocytes in mouse spleen is the experiment in which both R52.120 and R52.625 mAbs inhibited the IL-5-induced maturation of spleen cells to IgM-secreting cells (Table II). These types of experiments were performed with small, resting spleen cells purified by velocity sedimentation (16). However, since the population of IL-5-reactive B cells is small, we cannot completely exclude that some of the B cells that respond to IL-5 in this system have been preactivated *in vivo*. In the same experiment these mAbs did not inhibit the IL-2-induced maturation of the spleen B cells, while anti-IL-2-R mAbs did (Table II).

We have begun the biochemical characterization of the antigens recognized by the R52.120 and R52.625 mAb. These antibodies are likely to interact with the same or with sterically close epitopes on the IL-5-R as they block each other's binding to B<sub>13</sub> cells. We found that both antibodies specifically precipitate from cell lysates of <sup>35</sup>S-methionine-labeled B<sub>13</sub> cells three proteins with apparent *M<sub>r</sub>* of 46,000, 130,000, and 140,000 under reducing (Fig. 4) and nonreducing conditions (not shown), indicating that the three proteins are not linked by disulfide bridges. Similar results were obtained with another cell line that binds R52.120 and R52.625 mAbs (not

TABLE I  
*Precursor Frequencies of IL-5- and LPS-inducible IgM-Producing Cells  
in Total and R52.120<sup>+</sup> Spleen Cells*

Spleen cells	Precursor frequency of:	
	IL-5-inducible IgM-secreting cells	LPS-inducible IgM-secreting cells
R52.120 <sup>+</sup>	1:4–1:12	1:5–1:15
Total	1:60–1:200	1:11–1:18

The precursor frequencies of IL-5- and LPS-inducible IgM-secreting cells were determined as described (7). The percentage of positively sorted cells with mAb R52.120 varied from 1.5% to 2.5%. The results are the range of the frequencies obtained in four experiments.

TABLE II  
*R52.120 and R52.625 mAbs Block the IL-5-driven but not  
 the IL-2-induced Maturation of Small Resting C57BL/6  
 nu/nu Splenic B Cells to Antibody-secreting Cells*

Antibodies added to cultures*	B cell response stimulated by:	
	IL-2	IL-5
None	380†	425
R52.120	420	38
R52.625	360	47
PC61 + 7D4	25	410

Small resting B cells (at a concentration of  $5 \times 10^5$  cells/ml) were stimulated with 100 U/ml IL-2 or 5 U/ml IL-5. IgM-secreting cells were determined at day 5 of culture as described (7).

\* The mAbs R52.120 and R52.625 (10  $\mu$ g/ml) and the anti-IL-2-R antibodies (23, 24) (final concentration 10% hybridoma culture supernatant) were added at the beginning of the cultures.

† IgM-PFC/ $10^5$  plated cells.

shown). The immunoprecipitations of cell lysates from surface  $^{125}$ I-labeled  $B_{13}$  cells have not yet yielded an identifiable labeled protein, perhaps due to destruction of the R52.120 and R52.625 antigens by the iodination or because the proteins recognized by the mAbs are not efficiently iodinated.

The binding of radiolabeled IL-5 to its receptor followed by crosslinking of this complex have so far revealed only a single IL-5 binding protein of  $M_r$  45,000–50,000 (2, 10). On the basis of these molecular weight analyses, it is tempting to speculate that the IL-5 binding protein corresponds to the  $M_r$  46,000 molecule recognized by mAbs R52.120 and R52.625.

Our knowledge of the binding of IL-5 and the nature of the receptor identified by either IL-5 binding or mAb binding leaves us with at least two possible models for the IL-5-R. In the first model the IL-5-R is a complex of three proteins with  $M_r$  45,000–50,000, 130,000, and 140,000, in which  $M_r$  45,000–50,000 is the only IL-5 binding protein and  $M_r$  130,000 and 140,000 are possibly involved in signal transduction. Since IL-5 is biologically active only in its dimeric form (2, 10), low affinity binding would be the binding of IL-5 to one of these protein complexes,

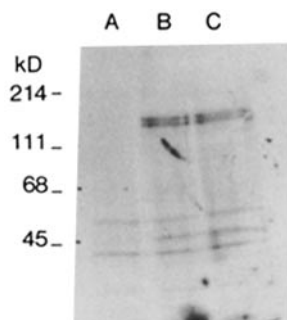


FIGURE 4. Biochemical characterization by SDS-PAGE of the antigen recognized by R52.120 and R52.625 mAbs. The cell lysates from  $^{35}$ S-methionine-labeled  $B_{13}$  cells were subjected to immunoprecipitation with control M41 (lane A), R52.120 (lane B), and R52.625 (lane C) antibodies. The autoradiogram represents a 10% gel, run under reducing conditions.

whereas high affinity IL-5 binding would represent the crosslinking of two of these complexes.

The finding that the monomeric biologically inactive form of IL-5 binds to the IL-5-R complex with low affinity and can block the high affinity binding of the dimeric form of IL-5 (unpublished results) supports this  $M_r$  45,000–50,000 homodimer binding model.

The second model would be that the IL-5-R is a "hetero" protein complex in which  $M_r$  45,000–50,000 is the IL-5 binding protein giving rise to only low affinity binding. For high affinity binding the IL-5 should have to interact with a second protein, maybe with  $M_r$  130,000 and/or 140,000. This second model would be comparable with the model for the IL-2-R. In the case of the IL-2-R, it is known that the presence of both p70 and p55 are necessary for binding of IL-2 with high affinity; p55 alone binds IL-2 with low affinity, while p70 alone binds it with intermediate affinity (22). Thus far, a second IL-5 protein has not yet been identified, and in particular, IL-5 binding to the  $M_r$  130,000 or 140,000 proteins has not yet been observed.

Ultimate proof for the nature of the IL-5-R will have to come from cloning of the genes encoding this receptor and subsequent functional tests by transfection experiments. The availability of monoclonal anti-IL-5-R antibodies and cell lines expressing high levels of IL-5-R with capacity of unlimited growth should now greatly facilitate this cloning as well as the study of the mechanism of action and the biological relevance of IL-5.

### Summary

The rat mAbs R52.120 and R52.625 inhibit the action of IL-5 on both IL-5-sensitive cell lines and freshly isolated splenic B lymphocytes. Neither antibody inhibits the proliferative cell responses promoted by IL-2, IL-3, or IL-4. Purified R52.120<sup>+</sup> lymphoid spleen cells contain 15–20-fold higher numbers of B lymphocytes responding to IL-5 in the form of maturation into antibody-producing cells. By immunofluorescence staining and flow fluorocytometry, the R52.120 and R52.625 antibodies bound to all 12 IL-5-sensitive cell lines tested. Both antibodies react with 2–4% cells in the spleen, 5% lymphoid cells, and 10–15% myeloid cells in the bone marrow, and 10–14% in the peritoneum of C57BL/6, DBA/2, and BALB/c adult mice. No positive cells for either antibody were detected in the thymus and lymph nodes of these mice. Both R52.120 and R52.625 antibodies specifically inhibit the binding of radiolabeled IL-5 to its receptor. Finally, R52.120 and R52.625 antibodies precipitate from <sup>35</sup>S-methionine-labeled IL-5-R<sup>+</sup> cell lysates three proteins with  $M_r$  46,000, 130,000, and 140,000. Taken together from these results, we conclude that the R52.120 and R52.625 mAbs recognize epitopes on the IL-5-R complex very close or identical to the IL-5 binding sites.

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