Immune responses to parasitic helminths are usually characterized by quite mysterious phenomena: dominance of Th2-like immunity and antigen-nonspecific IgE secretion. We previously purified a factor from *Dirofilaria immitis* that induces antigen-nonspecific IgE in rats and named it DiAg. In the presence of IL-4, DiAg induces mouse B cells to secrete IgE, which is antigen-nonspecific polyclonal antibody. We investigated the biochemical characteristics of DiAg as a factor of inducing IgE in this study. Recombinant DiAg (rDiAg) with interleukin (IL)-4 induced IgE synthesis in highly purified human normal B cells in vitro cell culture systems. The addition of recombinant human soluble CD40 IgG fusion protein (rsCD40-Ig) inhibited induction of IgE synthesis by rDiAg with IL-4. Monocyte cells were cultured with rDiAg and recombinant human soluble CD40L (rsCD40L); IL-12 and TNF-α were induced. The addition of rsCD40-Ig to THP-1 cells activated with rDiAg and rsCD40L inhibited the production of IL-12. rDiAg bound to the monocyte cell membrane fraction and recombinant human soluble CD40; this binding of rDiAg was competitively inhibited by addition of rsCD40L. Moreover, in CD40-deficient mice, IgE production and MLN-B cell proliferation by rDiAg were completely absent. Based on these results, we concluded that DiAg is an agonist of CD40.

Parasitic helminth infections are characterized by mast cell hyperplasia, eosinophilia (1), and markedly elevated levels of circulating antigen nonspecific IgE (2), which responses were concerned to helminth protection (3, 4). Parasitic antigen-specific IgE is associated with elimination of parasites from infected hosts, while antigen-nonspecific polyclonal IgE may be involved in the survival of invading parasites (3, 5, 6). We have previously reported that excretory-secretory component (native DiAg) isolated from *Dirofilaria immitis* induces both total IgE production in human spleen cells and CD23 expression on human splenic B cells in the presence of IL-4 (7). Lee et al. demonstrated that *Ascaris suum* pseudocelomic body fluid contains a B cell mitogen (8) and induces high levels of total IgE secretion. We previously purified a factor from *D. immitis* that induces antigen-nonspecific IgE in mice and rats and named it DiAg (10). DiAg was identical to one already identified as a polyradderprotein, or neutrophil chemotactic factor (DiNCF; GenBank accession number D88757) (11). To further characterize DiAg, we produced its recombinant protein, rDiAg, in *Escherichia coli* and analyzed its characteristics in a number of studies. rDiAg induces transmission of a mitogenic signal to mouse B cells, which in turn induces secretion of IgE in the presence of IL-4. Immune responses to parasitic helminths are usually characterized by two quite mysterious phenomena: dominance of Th2-like immunity and antigen-nonspecific IgE secretion. DiAg might cause these unique phenomena in the case of filarial infection. A number of critical questions about the mechanisms of polyclonal IgE synthesis remain to be resolved. First, it is not clear which molecules play an essential role in DiAg-induced activation of B cells. Second, the mechanisms by which IL-4 induces IgE secretion in helminth infection are not clear, although it has been established that the process is dependent on activated Th2 cells. T cell-dependent antigen-specific IgE synthesis in B cells requires that the signal be delivered by interaction of the B cell surface antigen CD40 with its ligand (CD40L) (12) expressed on activated T cells (13) with IL-4 or IL-13. CD40 is a 50-kDa glycoprotein expressed on B cells (14), dendritic cells, epithelial cells (15, 16), and monocytes (17). CD40L is directly bound to CD40 on B cells and induces mitogenicity in murine and human B cells (18, 19); it can also induce secretion of all immunoglobulin isotypes in the presence of cytokines (20). rDiAg has been previously shown to induce IgE secretion of splenic B cells from naive mice with IL-4 in vitro culture system in the absence of T cells. It suggests that the engagement of antigen-specific T cell to B cells is not involved in induction of IgE by rDiAg. DiAg might induce the IgE class switching via the CD40 molecule with the IL-4. We therefore investigated the biochemical characteristics of DiAg as a CD40 agonist. CD40L has been shown to induce cytokine production in peripheral blood monocytes and T cells (22). It is known that human monocyte-like THP-1 cells produce proinflammatory cytokines by CD40L stimulation (23). We have previously shown that rDiAg induces IgE production in rats. However, actions of rDiAg on human cell lines remain to be elucidated. We report here that rDiAg have various biological activity to the human cell lines and B cell population.

**EXPERIMENTAL PROCEDURES**

**Construction of the rDiAg**—The V1 domain (a repeating unit) of DiAg gene was amplified by polymerase chain reaction (PCR) with primers (5′-primer, including NdI restriction site: 5′-CGATCATATGATGATCATATAATTAGAAGGC-3′; 3′-primer, including BamHI restriction site: 5′-CTAAGGATCCCATCACCGGTACCAGGTCATATT-3′) from pDi6 (24), gifts from Dr. Makoto Owashi, Univ. of Tokushima, Tokushima, Japan), which code DiAg. Amplified DNA was digested with NdI and BamHI and cloned into pET3a vectors for expression in *E. coli* HMS174 (DE3). All constructs were verified by DNA sequencing. The purification of rDiAg was performed as follows. Five g of cell paste was

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suspended in 30 ml of 50 mM HCl and 5 mM EDTA at 4 °C and then centrifuged at 12,000 × g for 10 min. Recombinant DiAg in the supernatant was precipitated by 60–80% saturated ammonium sulfate and then applied on a Superdex 75 column. Contaminants of pyrogen from E. coli were removed from concentrated rDiAg solution by immobilized protein A. The isolated rDiAg protein was lyophilized and stored at −20 °C until use.

Construction of the Extracellular Domain of CD40—The extracellular domain of the human CD40 gene was amplified by PCR with primers (5′-primer: 5′-GGCTCATATGCAGAAAAACACCACTGC-3′, antisense primer: 5′-GGAGGCCAGGAAGATCGTGGGGAAA-3′) from cDNA (CLONTECH) of human leukocytes. Amplified DNA was digested with NotI and BamHI and cloned into pET3a vectors for expression in E. coli HMS174 (DE3). All constructs were verified by sequencing. The purification of rsCD40 was performed as follows. Five g of cell paste was suspended in 30 ml of lysis buffer (8 M urea, 100 mM Tris-HCl, pH 8.8) and sonicated by sonicator on ice. The lysate was centrifuged at 27,000 × g for 20 min. The supernatant was applied on a Mono-Q column (Amerham Pharmacia Biotech, 5 mm φ × 50 mm) preequilibrated with the same buffer. The active fraction was pooled and was precipitated by adding 80% saturated ammonium sulfate. The precipitate was washed in PBS and then applied on a Superdex 75 (Amersham Pharmacia Biotech, 10 mm φ × 300 mm) column chromatography preequilibrated with PBS. Contaminants of pyrogen from E. coli were removed from concentrated rsCD40 solution by immobilized polyoxymel B. Construction procedure of rsCD40-lg was performed as described by Hsu (25).

IgE Induction in Vivo and Measurement of Immunoglobulin Concentrations—Osmotic pumps (Alza) filled with rDiAg or ovalbumin in 100 μl of phosphate-buffered saline were subcutaneously injected in female 6-week-old mice (wild-type C57BL/6 or CD40-deficient B6.128P2-Tnfasf1ortm1kik; Jackson Laboratory stock number 002928). The pumping rate of an osmotic pump is 0.25 μl/h. Recombinant DiAg in the supernatant was harvested and assessed for their IgE content. In certain experiments, the sensitivity of the assay was 3 ng/ml. The level of IgE production was induced in rDiAg-infused mice, a significant amount of IgE detected in the supernatants of cycloheximide-treated rDiAg in normal B cells, we examined the effect of blocking CD40L-CD40 interactions. For this purpose, we used a recombinant soluble CD40-lg fusion protein (rsCD40-lg), which has been shown previously to inhibit IL-4-driven CD40L-depend-

A Factor of Inducing IgE from a Filarial Parasite

THP-1 or Ramos Cells—THP-1 or Ramos cells were cultured in 250 ml of culture medium (RPMI 1640 supplemented with 10% fetal calf serum) at 37 °C for 24 h in a humidified atmosphere of 5% CO2. The homogenized in buffer (5 mM Tris-HCl, pH 7.4, 2 mM EDTA, 5 μg/ml subtilisin inhibitor, 5 μg/ml leupeptin, 10 μg/ml benzamidine) by a politron homogenizer at maximum speed on ice. The homogenate was centrifuged at 45,000 × g for 30 min at 4 °C. The precipitate was suspended in buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 5 μg/ml subtilisin inhibitor, 5 μg/ml leupeptin, 10 μg/ml benzamidine) and the aliquots were stored at −80 °C until use. rDiAg and rsCD40-lg were radiolabeled with Bolton-Hunter reagent (diiodinate 163 TBq/mmol, PerkinElmer Life Sciences). Radiolabeled rDiAg and rsCD40-lg were purified by gel filtration. Membrane aliquots (40 μg of proteins) were incubated for 1 h at 4 °C with or without a protecting ligand in 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.2% bovine serum albumin, and 1 μM phenylmethylsulfonyl fluoride. Preincubated membranes were incubated with −0.98–31.2 nm 125I-rDiAg or 125I-TrasCD40-lg with or without competitor for 1 h at 4 °C. After incubation, radioligand binding membranes were filtered on Whatman GF/B filters by a cell harvester, and the filters were washed with phosphate buffer saline. The radioactivity of the filters was then determined in a γ counter (Wallac) 

Binding Assay for Membrane of THP-1 or Ramos Cells—THP-1 or Ramos cells were cultured in 250 ml of culture medium (RPMI 1640 supplemented with 10% fetal calf serum) at 37 °C for 24 h in a humidified atmosphere of 5% CO2. The same phenom-

Binding Assay for Membrane of THP-1 or Ramos Cells—THP-1 or Ramos cells were cultured in 250 ml of culture medium (RPMI 1640 supplemented with 10% fetal calf serum) at 37 °C for 24 h in a humidified atmosphere of 5% CO2. The same phenom-

RESULTS

IgE Induction in rDiAg-infused Mice—Serum immunoglobulin levels of C57BL/6J mice are shown in Fig. 1a. A significant level of IgE production was induced in rDiAg-infused mice, with peak IgE production occurring on day 21, and the level decreased gradually until day 28. In contrast, antigen-specific IgE levels were not detected at any time points measured (Fig. 1b). Our results were in agreement with the previous report of marked elevation of polyclonal IgE levels in the early phase of Nippostrongylus brasiliensis infections (5). The same phenomenon has also been observed in rats infected with N. brasiliensis.
Human B cell populations were purified from PBMCs in normal Ig. A Factor of Inducing IgE from a Filarial Parasite

**FIG. 1.** IgE levels of wild-type and CD40-deficient mice. Wild-type (circles) and CD40-deficient (triangles) mice were subcutaneously implanted with an osmotic pump (Alza) filled with rDiAg (closed symbols) or ovalbumin (open symbols), and serum were collected weekly. a, serum total IgE levels were measured by ELISA. Data are shown as the mean ± S.E. of five animals.* p < 0.01 by Student’s t test as compared with day 0 samples. b, antigen-specific IgE levels were measured with immobilized 1 μg/100 μlwell of rDiAg or ovalbumin by ELISA. Results of antigen-specific IgE were presented in means of absorbance at dilution of sera (1:50). Osmotic pumps filled with rDiAg or ovalbumin in 100 μl of phosphate-buffered saline and subcutaneously infused in female 6-week-old mice (C57BL/6 or CD40-deficient). The pumping rate of an osmotic pump is 0.25 μl/hr continuously, and the duration in 14 days. IgE concentrations of serum were measured by the ELISA kits (Bethyl). Antigen-specific IgE were measured by ELISA. One microgram of rDiAg or ovalbumin in 100 μl was coated onto a 96-well plate (Maxisorb, Nunc) at 4 °C for 16 h. The wells were then washed with PBS and blocked with milk casein (BlockA, Yukijirushi) for 1 h at 37 °C. After washing with PBS, 100 μl of diluted serum was added and was incubated for 1 h at 4 °C. After washing with PBS, bound antibody was detected by peroxidase-conjugated anti-mouse IgE (Nordic Immunological Laboratory).

**FIG. 2.** Inhibition of IgE synthesis in human B cells by rsCD40-Ig. Human B cell populations were purified from PBMCs in normal donors. B cells were stimulated with rsCD40L (2 μg/ml) or rDiAg (10 μg/ml) plus IL-4 (200 units/ml) for 14 days in the absence (open column) or presence of rsCD40-Ig (50 μg/ml; closed column) or control rsTNFR-Ig (50 μg/ml; gray column). IgE levels of the culture supernatants were measured by the ELISA kits. Results shown are representative of two experiments. Data are shown as the mean ± S.E. of triplicate assays. * p < 0.005 by Student’s t test as compared with the absence of rsCD40-Ig.

ent IgE synthesis in PBMCs (26, 27). Fig. 2 shows that rsCD40-Ig, but not the control recombinant human soluble TNF receptor I immunoglobulin fusion protein (rsTNFR-Ig; R&D Systems), inhibits induction of IgE synthesis by rsCD40L+IL-4 or rDiAg+IL-4. The enhancement of IgE synthesis observed with rsTNFR-Ig in the experiment presented was not reproducible, because it was not observed in other experiment. These results support the notion that induction of IgE isotype-switching by rDiAg+IL-4 is dependent on CD40.

**Production of Proinflammatory Cytokines by rDiAg Stimulation to THP-1 Cells**—The immune responses of human cell lines to the addition of rDiAg remain to be elucidated. In this study, therefore, we first examined the response of rDiAg to a human cell line. THP-1 cells secreted IL-12 upon activation by rDiAg, rsCD40L (Bender Medsystems), or LPS, as shown in Fig. 3a. Stimulation with 10 μg/ml rDiAg resulted in an up to 6-fold increase in secretion compared with that in non-treated controls. The addition of IL-10 to THP-1 cells stimulated with rDiAg rsCD40L, or LPS profoundly inhibited the production of IL-12. Phagocytosis of the cells was stimulated by addition of silica powder and hydroxyurea to the THP-1 cell culture medium, and TNF-α secretion was induced by rDiAg, rsCD40L, or LPS. As shown in Fig. 3b, the addition of IL-10 to LPS-activated THP-1 cells stimulation profoundly inhibited the production of TNF-α. In contrast, when THP-1 cells were activated by rsCD40L or rDiAg, the production of TNF-α was not significantly affected by the addition of IL-10. To demonstrate a casual relationship between the DiAg-induced and CD40-induced productions of inflammatory cytokines in THP-1 cells, rsCD40-Ig fusion protein (25) was added to the cell culture (Fig. 3c). The addition of rsCD40-Ig to THP-1 cells activated by rDiAg and rsCD40L stimulation inhibited the production of IL-12. In contrast, when THP-1 cells were activated by LPS, the production of IL-12 was not affected by the addition of rsCD40-Ig. Following addition of rsTNFR-Ig into the cell culture as a negative control, none of the stimulations of activated THP-1 cells studied had any effect on IL-12 production (data not shown).

**Binding Assay for THP-1 or Ramos Cell Membrane**—To investigate the effects of direct binding of rDiAg to cell membrane molecules, binding assays with 125I-rDiAg were conducted on THP-1 cells expressing human CD40. 125I-rDiAg bound to the THP-1 cell membrane fractions in a concentration-dependent manner (Fig. 4a). 125I-rDiAg binding to membrane fractions revealed a large number of receptors (Kd = 8.4 nM; 16,000 sites/cell) by Scatchard plot analysis (Fig. 4b). Binding of 225I-rDiAg to the THP-1 cell surface membrane was competitively inhibited by addition of rsCD40L and unlabeled rDiAg (Fig. 4c). IC50 by rsCD40L and rDiAg were 3.6 × 10−8 M and 2.4 × 10−7 M, respectively. To confirm the ability of rDiAg to competitively
inhibit 125I-rsCD40L binding to cell membrane molecules, binding assays with 125I-rsCD40L were conducted on Burkitt’s lymphoma Ramos cells expressing human CD40 (28). 125I-rsCD40L bound to the Ramos cell membrane fractions in a concentration-dependent manner (Fig. 4). 125I-rsCD40L binding to membrane fractions revealed the possible existence of two types of receptors (K_d high = 1.4 nM; 36,000 sites/cell and K_d low = 30 nM; 1.8 × 10^5 sites/cell) by Scatchard plot analysis (Fig. 4e). The high affinity of receptor was similar to the affinity of rDiAg binding. Binding of 125I-rsCD40L to the Ramos cell surface membrane was competitively inhibited by addition of unlabeled rsCD40L and rDiAg (Fig. 4f). IC_{50} by rsCD40L and rDiAg were 3.3 × 10^{-5} M and 3.8 × 10^{-7} M, respectively.

**Binding Activity of rDiAg and rsCD40L or TNF-α to rsCD40-Ig or rsTNFR-Ig—**The data described above suggest that DiAg activated the immune system by binding to CD40. However, there is a possibility that 125I-rDiAg binds to molecules other than CD40 because CD40 is a member of the TNF-α receptor family (29). We therefore attempted to determine whether or not rDiAg directly binds to rsCD40. The purified rDiAg and rsCD40L were immobilized on a 96-well plate and incubated with rsCD40-Ig and rsTNFR-Ig and then detected by incubating with anti-IgG Fc antibody. The specificity of binding was determined by competition with the excess of ligands added with the rsCD40L. The purified rDiAg and rsCD40L were able to bind rsCD40-Ig in an immobilized ligand concentration-dependent manner (Fig. 5a), but rDiAg and rsCD40L were not able to bind rsTNFR-Ig (Fig. 5b).

**IgE Levels of Wild-type and CD40-deficient Mice—**To confirm whether the IgE-inductive and cell proliferation effects were due to CD40 agonist properties of DiAg, we compared the effects of DiAg in wild-type and CD40-deficient mice (30) (Strain Name: B6.129P2-Tnfrsf5tm1Kik, Jackson Laboratory). The serum immunoglobulin levels of control and CD40-deficient mice are shown in Fig. 1. Basal serum levels of IgE in CD40-deficient mice were not significantly different from those in wild-type mice (Fig. 1a; day 0). CD40-deficient mice were infused with rDiAg or ovalbumin as a control. Total IgE production of wild-type mice was already significant at 14 days and was maximal at 21 days after administration of rDiAg (Fig. 1a). On day 21 after administration, total and specific IgE production of ovalbumin was slightly increased, but DiAg-specific IgE production was not detected (Fig. 1b). In contrast, total IgE production of CD40-deficient mice was completely absent.

**CD40-deficient Mice MLN-B Cell Proliferative Responses—**MLN-B cells were purified from CD40-deficient mice or wild-type mice to assess their ability to respond to stimulation of DiAg in vitro. As shown in Table I, MLN-B cells from CD40-deficient and wild-type mice exhibited comparable proliferative responses to LPS. MLN-B cells from wild-type mice stimulated with rDiAg showed enhanced proliferative responses. In contrast, MLN-B cells from CD40-deficient mice stimulated with rDiAg did not show such enhanced proliferation. It is known (30) that B cells from CD40-deficient mice and wild-type mice exhibited commensurable proliferative responses to LPS, anti-IgM, and IL-4, although only CD40L did not respond to B cell from the CD40-deficient mice. This result resemble to our results of MLN-B cells responses by DiAg.

**DISCUSSION**

Dendritic cells are professional antigen-presenting cells that are specialized in the initiation of T cell-dependent immune responses. It is known that human dendritic cells (DC) secrete high levels of TNF-α and IL-12 upon either activation by lipopolysaccharide (LPS) or CD40 engagement (31). The addition of IL-10 during DC stimulation profoundly inhibited the production of the cytokines by LPS-activated DC (32). In contrast, when DCs were activated by co-culture with CD40L transfectants, TNF-α were not significantly affected by the addition of IL-10, but IL-12 levels were markedly decreased (32). It is also known that antigen-presenting THP-1 cell lines produce proinflammatory cytokines (23) such as IL-12 and TNF-α by CD40L stimulation. We found that rDiAg directly stimulated a human immune cell line, THP-1 cell, and induced proinflammatory cytokine production. When THP-1 cells were stimulated with rDiAg or recombinant human soluble CD40L (rsCD40L), IL-12 and TNF-α were induced. These results demonstrate that human antigen-presenting cells show similar responses to both CD40L and rDiAg. And the production of IL-12 was inhibited by addition of recombinant human soluble CD40 (rsCD40). rDiAg-induced cytokine production was not due to contamination of LPS in the preparation of recombinant protein for the
following three reasons. First, the level of LPS within rDiAg, as revealed by Endotoxin Test-D (Seikagaku Co., Ltd., Tokyo, Japan), was below the detectable limit (sensitivity: 0.0058 enzyme units; 2 pg/ml). Second, C3H/HeJ mice, which are nonresponsive to LPS, responded to rDiAg as well as wild-type C3H/HeN mice (data not shown). Third, rDiAg activity was not affected by addition of polymixin B, an inhibitor of LPS (data not shown).

rsCD40L competitively inhibited rDiAg binding to the membrane of these cells. $^{125}$I-rDiAg or $^{125}$I-rsCD40L bound to the cell membrane. Cell membranes were incubated with $^{125}$I-rDiAg or $^{125}$I-rsCD40L. After incubation, radioligand binding membranes were filtrated on Whatman GF/B filters, and filters were washed with phosphate buffer saline. Data are shown as the mean ± S.E. of triplicate assays. Similar results were obtained in two separate experiments.

**FIG. 4.** Binding assay for THP-1 or Ramos cell membrane. Binding assays with $^{125}$I-rDiAg (a, b, and c) or $^{125}$I-rsCD40L (d, e, and f) were conducted on THP-1 or Ramos cells expressing human CD40 respectively. a and d, typical curves for the binding of $^{125}$I-rDiAg or $^{125}$I-rsCD40L to the cell membrane. Cell membranes were incubated with $^{125}$I-rDiAg or $^{125}$I-rsCD40L. After incubation, radioligand binding membranes were filtrated on Whatman GF/B filters, and filters were washed with phosphate buffer saline. Data are shown as the mean ± S.E. of triplicate assays. Similar results were obtained in two separate experiments.
bound directly to rsCD40. rDiAg was not affected in CD40-deficient mice. The experimental evidence (30) with the CD40-deficient mice showed that the in vivo T cell-dependent immunoglobulin class switching but not the T cell-independent antibody responses is affected. Our results showed that polyclonal IgE induction by DiAg was affected in CD40-deficient mice. It means that DiAg induced polyclonal IgE by the mechanism that is not the T cell-independent antibody responses. If DiAg induces the class switching by the signals other than via CD40 molecules, DiAg induces polyclonal IgE production in CD40-deficient mice. Therefore, DiAg might induce the IgE class switching via the CD40 molecule with the IL-4. We concluded that DiAg is an agonist for human CD40. DiAg nonspecifically activates B cells through CD40 and secretes a large amount of polyclonal IgE antibodies. Antigen-nonspecific polyclonal IgE may be involved in the survival of the invading parasitic helminth. On the other hand, DiAg might interfere with the antigen-specific helper T cell activation by the CD40-CD40L binding. However, IL-4 is necessary for the IgE class switching and the differentiation into the Th2 cells, and the activation by DiAg are indispensable. DiAg might stimulate IL-4 production by Th2 cells in an as-yet-undefined manner. Armitage (22) et al. demonstrated that, in addition to its stimulatory effects on B cells and monocytes, CD40L costimulates the proliferation of activated T cells. This response is accompanied by the production of IFN-γ, TNF-α, and IL-2 but is largely IL-2 independent. These data demonstrated that CD40L is a T cell stimulatory factor, which may play an important role in interaction between T cells following activation through helminth infection. As an agonist of CD40, DiAg might be a T cell-stimulatory factor in the same manner as CD40L. In fact, low-level CD40 expression has previously been reported on some human T cell lines and gibbon T cell lymphomas (33–35). During its several hundred million years of coexistence with a host, the helminth may have acquired the means to escape attack from the host immune system. To escape the host immune system, the parasitic helminth might have acquired the analogue of CD40L. As for the homologues of DiAg (36–40), the DNA and amino acid sequence is conserved in many families of helminth. These homologues are called polyproteins, which consist of repeating units including a cluster of tetra-basic amino acid sequences. The repeating units of all polyproteins might have agonistic effects for CD40. In fact, we found that a recombinant ABA-1 (Ascaris lumbricoides polyprotein) has similar biological activity to rDiAg (data not shown). The preservation of a similar protein by numerous species might indicate that the protein plays an important role. It has been reported that ABA-1 has other biological activities, including binding to small lipids (40). It is not known whether the binding to the lipid induces the production of IgE in B cells, or whether it induces the cytokine production in monocyte cell lines. The maximum Kd value of binding to the lipid of recombinant ABA-1 is about 1 μM, and its binding affinity is low. Such binding power is not sufficient to explain the specific biological activity on the part of this polyprotein. The binding of rDiAg to CD40 occurs with relatively high affinity and could explain the specificity of the immune responses by rDiAg. The homology between the amino acid sequences of DiAg and sCD40L is not high. In the isoelectric point forecast from the amino acid sequence, rDiAg was 8.5 and rsCD40L was 9.5. There was little similarity in the presumable secondary structure between the two peptides (data not shown). It has been reported that the soluble recombinant form of CD40L has a

### Table 1

| Stimulus | Wild-type % | CD40-deficient % |
|----------|-------------|------------------|
| None     | 100.0 (0.9) | 100.0 (1.0)      |
| rDiAg 3 μg/ml | 179.3 (2.4) | 95.1 (2.0)      |
| rDiAg 10 μg/ml | 251.7 (1.4) | 99.5 (1.4)      |
| LPS 10 μg/ml  | 613.8 (2.6) | 175.1 (2.3)    |

**FIG. 5. Binding activity of rDiAg and rsCD40L or TNF-α to rsCD40-Ig or rsTNFR-Ig.** Serial dilutions of purified rDiAg, rsCD40L, and TNF-α were coated onto a 96-well plate (Maxisorb, Nunc) at 4°C for 16 h. The wells were then washed with PBS and blocked with milk casein (Block-A, YukiJirushi) for 1 h at 37°C. After washing with PBS, 100 μl of rsCD40-Ig (2 μg/ml diluted in 0.1% bovine serum albumin in PBS) was added and was incubated for 1 h at 4°C. After washing with PBS, bound antibody was detected by peroxidase-conjugated anti-IgG Fe antibody (Jackson). α, this figure shows the binding of rsCD40L (open circles) and rDiAg (closed circles) to rsCD40-Ig as described under “Experimental Procedures.” Curves represent the percentage of maximum binding of rsCD40L to rsCD40-Ig. Data are shown as the mean ± S.E. of triplicate assays. Similar results were obtained in two separate experiments. b, this figure shows the binding of rsTNF-α (open triangles) and rDiAg (closed circle) to rsTNFR-Ig. Curves represent the percentage of maximum binding of rDiAg to rsTNFR-Ig. Similar results were obtained in two separate experiments.
trimeric structure (21) and that the three-dimensional structure of rDiAg might be similar to the trimeric structure of sCD40L. It is known that the anti-CD40 antibody often shows agonistic activity. Such foreign proteins as anti-CD40 antibodies and rDiAg might show agonistic activity, since the ligand recognition of the CD40 molecule is a little vague. The similarity of the responses of DiAg between humans and rodents shows that specificity in the interspecies of CD40 is low. It is not unusual for numerous species to share in common a molecule that serves a similar function. We hypothesize that DiAg is a molecule newly acquired to maintain the species by the process of evolution within the special environment of parasitism. The potential role of the CD40/CD40L engagement is very important, and many immunologists have been engaged in the study of this molecule in recent years. The helminth can be said to have recognized the importance of this molecule for several hundred million years. And indeed, it is quite a feat to have acquired such a molecule for the purpose of evading the host immune system. The parasitic helminth may have acquired the ability to escape the host immune system through evolutionary acquisition of CD40 agonist.

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