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Early Local Generation of C5a Initiates the Elicitation of Contact Sensitivity by Leading to Early T Cell Recruitment[1]

Ryohei F. Tsuji,[2]* Ivana Kawikova,[†] Rajani Ramabhadran,[†] Moe Akahira-Azuma,[†] Dennis Taub,[‡] Tony E. Hugli,[§] Craig Gerard,[¶] and Philip W. Askenase[3†]

We have shown previously that an early complement C5-dependent cascade is required to recruit T cells to elicit 24-h contact sensitivity (CS) responses. In this paper, we have characterized molecular events of this early required cascade by biochemically analyzing extracts of mouse ears undergoing elicitation of CS. Chemotactic activity was found after local Ag challenge, in CS ear extracts early (by 1 h), in CS ear extracts late (through 24 h), in previously immunized mice, but not in ears of vehicle-immunized or non-immune-challenged mice. The early chemotactic activity at 2 h was likely caused by C5a, because it was neutralized in vitro by anti-C5a Ab, was inactive on C5aR-deficient (C5aR$^{-/-}$) macrophages, and was absent in C5-deficient mice. The activity was present in T cell-deficient mice, but elaboration was Ag-specific. This T cell-independent, Ag-specific elaboration of C5a early in CS ear responses likely led to T cell recruitment, because subsequent local IFN-γ mRNA and protein expression, as markers of T cell arrival and activation, began by 4 h after Ag challenge. In contrast to early C5a chemotactic activity, late chemotactic activity 24 h after Ag challenge was unaffected by anti-C5, was active on C5aR$^{-/-}$ macrophages, was T cell-dependent, and by ELISA appeared largely due to chemokines (macrophage-inflammatory protein-1α and -1β, IFN-γ-inducible protein-10, and monocyte chemoattractant protein-1). Importantly, early generation of C5a was required for T cell recruitment because C5aR$^{-/-}$ mice had absent 24-h CS. Taken together, these findings indicate an important linkage of C5a as a component of early activated innate immunity that is required for later elicitation of acquired T cell immunity, probably by facilitating the initial recruitment of T cells into the Ag-challenged local site in CS responses. *The Journal of Immunology,* 2000, 165: 1588–1598.

Contact sensitivity (CS)[4] is a form of delayed-type hypersensitivity (DTH) and is a classical example of in vivo T cell immunity. CS is induced by topical cutaneous sensitization with reactive haptens and is elicited subsequently by local skin challenge with the same hapten (1). Ag/MHC-restricted CD4$^+$ Th1 cells (1), or CD8$^+$ Tc1 effector cells (2), mediate CS inflammation that peaks at 24 h by recruiting mononuclear leukocytes and granulocytes to the site of Ag challenge. T cell-derived IFN-γ is a key cytokine in local expression of this 24-h inflammation in DTH (3). Because T cells are essential and very few Ag-specific recruited T cells can mediate DTH (4), an important

question for understanding and possibly manipulating CS and similar T cell DTH responses concerns how the few essential T cells themselves are recruited into local Ag-challenged sites. There are several possible pathways for this initial T cell recruitment to elicit CS. Traditionally, T cells are thought to survey the circulation and tissues until they encounter Ag peptides on APC (5). Accordingly, T cells would randomly migrate into tissues, but it was clearly demonstrated recently that activation of endothelial cells is needed for movement of T cells into extravascular tissues at the site of Ag challenge (6).

Endothelial activation needed for T cell recruitment in CS involves both Ag-nonspecific events and Ag-specific events. These processes act in an early and required CS-initiating phase that follows soon after local Ag challenge to elicit CS. Nonspecific local irritation by the chemically reactive hapten Ag was suggested to recruit T cells in CS by activating keratinocytes to produce endothelial-activating cytokines, such as IL-1 and TNF-α (5, 7). However, in contrast, we demonstrated an Ag-specific early cascade reaction that was required for subsequent elicitation of 24-h CS in mice challenged 4 days after sensitization (8, 9). This Ag-specific CS-initiating cascade was activated just within 2 h after Ag challenge (10). Participating components in this Ag-specific CS initiation process were mast cells (10), platelets (11, 12), their release of serotonin (5-HT) (10–13), which is the crucial vasoactive amine in mice (14), and also TNF-α (15, 16), leading to local activation of the endothelium. Thus, it was shown recently in murine CS that early Ag-specific release of TNF-α induced expression of VCAM-1 and ICAM-1 on the luminal surface of local endothelium, beginning at 4 h after Ag challenge (17). Prior release of TNF-α by skin mast cells probably mediated this local expression of vascular adhesion molecules by 4 h, because skin injection of TNF-α itself caused expression of these adhesion molecules within 2 h (17).

Recently, we showed that complement (C) also is involved in CS initiation (18, 19). C is known to be a major component of acquired B cell effector immunity, to play a role in regulation of B cell immunity (20, 21), and to participate in innate immunity (20). Although C has received little attention in acquired T cell immunity, our recent data suggest that C could participate in CS and DTH (18, 19). We showed that congenitally C5-deficient mice have impaired CS, which was restored by reconstitution with C5 (18), and that anti-C treatment with anti-C5 mAb or with C-inhibitory soluble C receptor-1 (sCR1), inhibited CS and DTH responses. Inhibition of CS by these C-inhibiting agents was demonstrated by decreased ear swelling, cellular infiltration, and impaired elaboration of chemotactic activity in 24-h CS ear extracts. Inhibition of CS required that C-inhibitors were given before Ag challenge (19), whereas they were ineffective if given just after the early component of CS (19), suggesting that C5 was activated early in elicitation of CS. Also, we showed that B cells were involved in CS, because CS was impaired in B cell-deficient μMT mice (19). Taken together, these findings suggested that early after Ag challenge, C could be activated locally via Abs to produce a proinflammatory mediator, such as C5a, that might directly or indirectly lead to recruitment of T cells. Thus, the major question of the current study about CS-initiating events early during elicitation of CS was whether local activation of C5 to elaborate C5a could play a role in early T cell recruitment.

To answer these questions we needed a way to directly demonstrate activation of C at the CS challenge site early after elicitation with Ag. Commonly used approaches to similar questions, such as knock-out mice, transgenic mice, mAb treatment, drug blocking, or correlative in vitro assays, were not sufficient to answer these questions. These techniques only allow measure of the final outcome of a CS response, which usually is evaluated 24–48 h after Ag challenge by macroscopic ear swelling and by microscopic cell infiltration. In contrast, it is hard to directly study the early CS initiation processes that are needed for eliciting subsequent 24–48 h CS, because the early phase has no cell infiltration (22) and is present as a smaller and transient ear swelling that peaks at 2 h (8–10). Thus, we previously had identified separate components of the early CS-initiating phase by determining ear swelling at 2 h after Ag challenge or indirectly by measuring the resulting 24-h ear swelling (10–13).

Recently we employed a new approach to determine the biochemical events in CS by preparing extracts from ears undergoing CS responses, and we identified and quantified molecules by sensitive in vitro assays (19). Thus, we analyzed the ear extracts for their chemotactic activity to attract the J744A.1 macrophage cell line that is rich in C5a receptors (C5aR; CD88), because they migrated strongly to C5a and had no chemotactic activity to C5a-deficient zymosan-activated mouse serum (ZAMS), which was prepared from C5-deficient mice. In this current study, we established that the early phase of CS is mainly C5a-dependent and that C5a is an essential molecule in early CS initiation that is needed for early recruitment of the CS effector Th1 cells, to then be activated to produce IFN-γ. The exact role of C5a in the CS initiation process is uncertain. It is possible that C5a may act directly as a chemotactic factor for the T cells (23) or to activate endothelial C5aR (24, 25). However, the current data combined with prior studies (10–14, 17) favor that C5a elaborated early after challenge in CS ears likely triggers C5aR on local mast cells to release TNF-α (26, 27) and also activates C5aR on platelets and mast cells to release 5-HT (28–30). Together, these C5a-released vasoactive mediators probably lead to local endothelial activation and expression of adhesion molecules, which is crucial for local early T cell recruitment in CS (17, 22).

Materials and Methods

Mice

Specific pathogen-free male CBA/J, DBA/1, DBA/2, B10.D2/n, and B10.D2/o mice (6–8 wk old) were obtained from The Jackson Laboratory (Bar Harbor, ME) and were rested for at least 1 wk before use. The DBA/2 and B10.D2/o mice were congenitally C5-deficient. $\alpha\beta$TCR-deficient (TCR$^{-/-}$; bred >10 generations with BALB/c), $\gamma\delta$TCR-deficient (TCR$^{-/-}$; bred >10 generations with C57BL/6) (both from Adrian Hayday, Yale University, New Haven, CT), and C5aR (CD88)-deficient (C5aR$^{-/-}$) mice (31) and 129/B6 controls were bred and maintained in filter-topped microisolator cages in a bioclean room and were fed autoclaved food and water. All experiments were conducted according to guidelines of the Animal Care Committee of Yale University School of Medicine.

Reagents

Picryl chloride (PCl; Nacalai Tesque, Kyoto, Japan) was recrystallized twice and stored protected from light. Human recombinant C5a was purchased from Sigma (St. Louis, MO). Both 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (OX) and Plummer's reagent (DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid) were obtained from Aldrich (Milwaukee, WI) and Calbiochem (La Jolla, CA). Rabbit anti-murine macrophage-inflammatory protein (MIP)-1α, MIP-1β, and monocyte chemoattractant protein-1 (MCP-1) Abs and rabbit anti-human IFN-γ-inducible protein-10 (IP-10) Abs were prepared by multiple-site immunization of New Zealand White rabbits using recombinant murine chemokines in IFA. Polyclonal Abs were titered by ELISA, and specificity was verified by examining cross-reactivity with other murine and human chemokines. Anti-human IP-10 antiserum was cross-reactive with murine IP-10.

Immunization, elicitation, and in vivo evaluation of CS responses

Mice were contact sensitized with 100 μl of 5% PCl or 3% OX in absolute ethanol and acetone (4:1) on the shaved chest and abdomen. Four days later, CS responses were elicited by painting both ears with a topical application of 10 μl of 0.4% or 0.8% PCl in acetone and olive oil (1:1). In some experiments, a lower dose of 0.4% was chosen, compared with the higher conventional eliciting dose of 0.8% PCl, because effects of C alterations on CS responses usually were observed with a moderate challenge dose (18). Mice on a C57BL/6 and C57BL/10 background produce inferior responses to the above PCl contact sensitization procedure, which is optimal for CBA and BALB/c. Thus, B6 background C5aR$^{-/-}$, 129/B6, B10.D2/n, and B10.D2/o mice produced suboptimal sensitization with our standard procedure. Therefore, to achieve more optimal CS response in these strains, they were sensitized twice on days 0 and 1 and were challenged with 0.8% PCl in acetone and olive oil (4:1) on day 7. Resulting thickness of the Ag-challenged ears in all strains was measured with a dial caliper (Ozaki, Tokyo, Japan) before challenge and then at 2 h and 24 h after challenge. Increases in ear thickness in groups of 4–6 mice were expressed as the mean $\pm$ SE $\times$ 10^{-3} cm.

Soluble TNF-αR (sTNFR), a fusion protein of dimeric human TNF-αR p80 and non-complement-fixing human IgG$_1$ Fc, which binds murine TNF-α, was used to treat mice either before or 3 h after PCl ear challenge, to see whether TNF-α was involved in the early or the late phase of elicited CS responses. The sTNFR (rhu TNFR:Fc; Immunex, Seattle, WA) was injected i.p. at a dose of 250 μg per mouse 30 min before or 3 h after 0.8% PCl challenge on the ears of previously 5% PCl immunized CBA/J mice. Controls received saline containing 250 μg human IgG$_1$ (Sigma).

In vitro measurement of chemotactic activity in CS ear extracts

Chemotactic activity was measured in extracts of three 4-mm punch biopsies per ear collected from the distal site of CS ear responses (19). The biopsies were frozen together rapidly in liquid N$_2$ and were subsequently thawed and extracted in 300 μl cold PBS on ice with a tissue microhomogenizer (Biospec Products, Racine, WI). Separate supernatants from four to five mice per group were diluted two to four times in RPMI 1640 with 1% gelatin without serum (RPMI-gelatin) and placed in lower 96 chemotaxis wells (Neuro Probe, Cabin John, MD). Target J774A.1 macrophages were suspended in RPMI-gelatin at 2–5 $\times$ 10^6 cells/ml, and 50 μl was added to upper wells, allowing migration through a polyvinyl pyrrolidone-free polycarbonate filter with 5- or 8-μm pores at 37°C for 4 h under 5% CO$_2$. In experiments with C5aR$^{+/+}$ vs C5aR$^{-/-}$ mice, peritoneal exudate macrophages were induced by i.p. injection of 2 ml thyoglycolate broth. Peritoneal lavage on day 3 consisted of about 95% macrophages (32). Migrated macrophages attached to the lower surface of the filter were

fixed, stained with Diff Quick (Kokusai Chemicals, Tokyo, Japan) and counted at five different filter spots from each well, or each filter was extracted with 4 M urea before measurement of absorbance at 650 nm. J774A.1 migration was not due to chemokinesis because addition of ear extracts to upper wells, where the cells were loaded, caused diminished migration.

Quantitative sandwich ELISA for IFN-γ and chemokines

Two specific anti-IFN-γ mAbs (PharMingen, San Diego, CA) were employed (33). Briefly, wells were coated overnight with 2 μg/ml capture anti-IFN-γ mAb (R4-6A2) in 0.1 M NaHCO₃ (pH 8.3) at 4°C. After blocking with 1% BSA in PBS at 25°C for 2 h, ear samples and recombinant mouse IFN-γ (Genzyme, Cambridge, MA) were added and incubated for 1 h at room temperature. Then, 1 μg/ml of another biotinylated anti-IFN-γ mAb (XMG1.2) and 1:3000 diluted HRP-conjugated streptavidin (Vector, Burlingame, CA) were added to detect IFN-γ. Then, tetramethylbenzidine (TMB), peroxidase substrate, and TMB one component stop solution (Kirkegaard & Perry, Gaithersburg, MD) were used for color development at 450 nm.

The levels of murine MIP-1α, MIP-1β, MCP-1, and IP-10 in ear extracts were measured by specific ELISA as described (34). Similar ELISAs are not yet available for mouse C5a. Briefly, microwells were coated with 1 μg/well of affinity purified rabbit anti-mouse MIP-1α, MIP-1β, MCP-1, or IP-10 Abs for 18 h at 4°C. After blocking with 3% BSA in PBS for 120 min at 37°C, 50 μl of ear extracts and recombinant chemokines (R&D Systems, Minneapolis, MN) was added and incubated for 1 h at 37°C. Then, 50 μl of biotinylated polyclonal rabbit anti-mouse chemokine Ab and 50 μl of a peroxidase-conjugated streptavidin (Bio-Rad, Richmond, CA) were added to incubated to detect chemokines. Chromogen substrate (Bio-Rad) was added and incubated at room temperature until optimal development was observed. The reaction was terminated with 50 μl of 2 M H₂SO₄ solution, and the plates were read at 490 nm on a Bio-Rad ELISA reader.

RNA isolation

Naive, vehicle-painted, and PCl-sensitized CBA/J mice had ears removed at various times after local PCl challenge that were flash-frozen in liquid N₂ and stored at −70°C until use. For ear RNA extraction, Trizol reagent (Life Technologies, Rockville, MD) was added to the frozen ears pooled from three mice/group, and tissue homogenate was obtained using Polytron electric homogenizer. RNA was purified per manufacturer's instructions as a modification of the single-step RNA isolation method (35) and then was dissolved in diethyl pyrocarbonate-treated, autoclaved, double-distilled H₂O and stored at −70°C.

RT-PCR and gel electrophoresis

For first strand synthesis of cDNA, 1 μg total RNA was reverse transcribed in a reaction mixture (25 μl of: 50 mM Tris-HCl (pH 8.3); 75 mM KCl; 3 mM MgCl₂; 10 mM DTT; 0.5 mM each of dATP, dCTP, dGTP, and dTTP (Pharmacia, Piscataway, NJ); 20 μg/ml oligo d(T)₁₅ (Promega, Madison, WI); 1 U/ml RNasin (Promega); and 8 U/ml M-MLV Reverse Transcriptase (Promega)) at 37°C for 45 min and then at 99°C for 1 min for one cycle before soaking at 4°C and using a Perkin-Elmer (Norwalk, CT) Thermocycler. Amplification of IFN-γ-specific cDNA was conducted on a 1/10 aliquot of the ss-cDNA (25 μl of 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 2.5 mM MgCl₂, 250 μM dNTPs, 0.8 mM each of forward and reverse primers (human, mouse, and rat IFN-γ specific amplimer set; Clontech Laboratories, Palo Alto, CA), and 1 U/ml *Taq* polymerase in storage buffer A (Promega)). Amplification of the housekeeping G3PDH cDNA was similar, using 1/25 aliquot of the ss-cDNA and Clontech's mouse G3PDH-specific amplimer set. Amplification cycles were as follows: one cycle of 94°C for 2 min; then 30 cycles of 94°C for 40 s, 60°C for 20 s, and 72°C for 25 s; and then one cycle of 72°C for 5 min. Finally, samples were soaked at 4°C. The amplified products were electrophoresed on a 1.8% agarose gel in Tris-acetate-EDTA buffer and viewed by ethidium bromide staining.

Statistics

Statistics were performed using the paired two-tailed Student *t* test, and *p* < 0.05 was taken as the level of significance.

Results

IFN-γ in CS ear extracts is a marker for the onset of T cell recruitment

Th1 and Tc1 cells mediate cellular immunity in DTH by producing IFN-γ locally (3, 36). To biochemically identify CS-initiating events occurring before the onset of the late 24-h T cell component of CS, we performed a study to detect the time course of IFN-γ levels in ear extracts. A large amount of IFN-γ was found by ELISA at 24 h (Fig. 1*a*). In addition, a small amount of IFN-γ was first detectable as early as 4 h after elicitation of CS by painting the PCl Ag on the ears of mice that were contact-sensitized with PCl 4 days before. In contrast, no increased IFN-γ was found in ear extracts of similarly PCl-challenged control mice that were sham-immunized previously by painting on the abdomen with the vehicle alone (Fig. 1*a*). In addition, assay of IFN-γ mRNA, determined by RT-PCR on the ear extracts, confirmed that IFN-γ was detectable in sensitized and challenged mice at 4 h through 24 h after local application of PCl, but not at 2 h and not in sham-sensitized controls that were challenged similarly with PCl (Fig. 1*b*).

This 4-h time point is just when mast-cell-dependent TNF-α begins to induce expression of endothelial adhesion molecules (17), which likely is needed for local T cell recruitment across the vessels to then be activated by Ag/APC in the tissues to produce the IFN-γ. Fig. 1*c* confirms that TNF-α probably was involved in this early initiating phase of CS. Treatment of PCl-sensitized CBA/J mice with a sTNFR just before PCl ear challenge significantly reduced the 2-h and 24-h components (group D vs group C). In contrast, similar sTNFR treatment beginning at 3 h after challenge, and thus just after the early initiating component of CS, had no effect on the 24-h component (group E vs group C). These results suggest that TNF-α was released during the early initiating phase of CS, because treatment with the sTNFR after the 2-h peak of macroscopic CS initiation had no effect.

Elaboration of IFN-γ mRNA in CS ear extracts at 4 and 24 h required αβ-T cells because sensitized and challenged TCR⁻/⁻ mice, compared with sensitized and challenged TCR⁺/⁺ mice, had no IFN-γ detected by RT-PCR at 4 h (Fig. 1*d*) and no IFN-γ detected by ELISA of CS ear extracts at 24 h (Fig. 1*e*). Because prior studies indicated that CD4⁺ MHC class II-restricted T cells mediated CS in the PCl model of CBA mice that we employed (37), we concluded that local IFN-γ levels at 24 h could be used as a marker for the recruitment and subsequent local activation of effector αβTCR⁺ Th1 cells in CS ears. In addition, the data indicated that early release of TNF-α led to recruitment and then activation of Th1 cells, such that local measurements made in the ears prior to 4 h were part of CS initiation and therefore occurred before and did not depend on T cells or on their elaborated IFN-γ.

Early generation of local chemotactic activity in CS responses

We analyzed chemotactic factors in ear extracts from the early phase of CS that might eventually lead to T cell recruitment to produce IFN-γ in the late phase. We previously have shown that elaboration of chemotactic activity at 24 h in CS ears was C- and C5-dependent, because injection of C-inhibitory sCR1 and anti-C5 mAb before Ag challenge inhibited the 24-h chemotactic activity in CS ears (19). Importantly, these prior studies suggested that C activation actually occurred early after local Ag challenge (within 3 h), because when sCR1 was injected systemically just 3 h after Ag challenge, there was no inhibition of 24-h CS (19). Because CS was decreased in C5-deficient mice (18) and late phase IFN-γ was blocked by anti-C5 mAb (19), we hypothesized that local chemotactic activity was a result of C5-derived C5a, which is a powerful chemotactic factor. Thus, we employed a chemotactic assay with J77.4A1 indicator cells that highly express C5aR and thus detect C5a by its chemotactic activity. Fig. 2 shows a time course analysis of chemotactic activity in CS ear extracts after Ag challenge. Chemotactic activity was present as early as 1 h after Ag challenge of sensitized mice, clearly near the onset of the early phase. Fig. 2 also shows chemotactic activity at each time point after 0.8% PCl
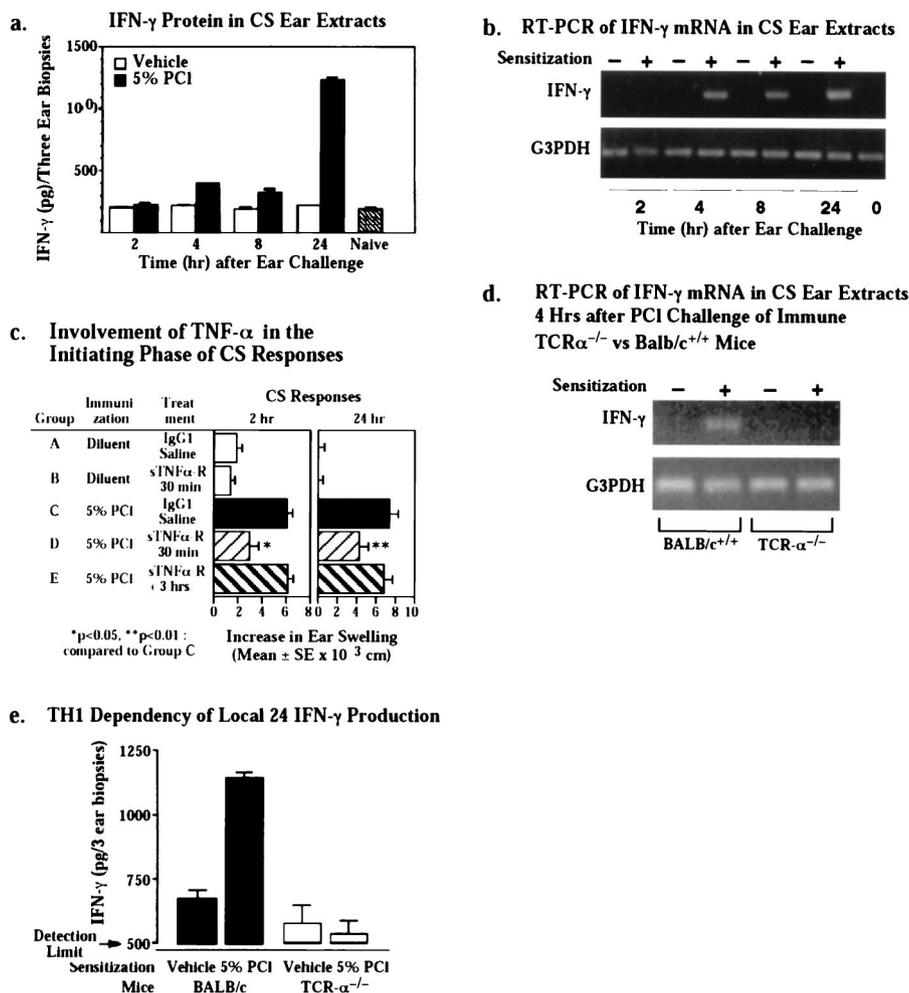
FIGURE 1. $\alpha\beta$-T cell-dependent elaboration of IFN-γ at CS sites and early involvement of TNF-α. *a*, CBA/J mice were contact sensitized on day 0 with 5% PCl or vehicle (ethanol:acetone = 4:1). On day 4, all mice were challenged to elicit CS by painting both ears with 0.8% PCl in acetone:olive oil (1:1). At the indicated times after Ag challenge, ear extracts were prepared (see *Materials and Methods*) to determine IFN-γ levels in CS ears by ELISA. In some groups, error bars were too small to observe. *b*, RT-PCR was used to determine IFN-γ mRNA in CS ear extracts obtained at different times after Ag challenge. G3PDH mRNA was used as control for transcripts of a housekeeping gene. *c*, Involvement of TNF-α in the initiating phase of CS responses. A sTNFR was used as TNF-αR antagonist and was given either just before PCl ear challenge of PCl-sensitized mice (*group D*) or 3 h after PCl challenge of similar mice (*group E*) and compared with similarly PCl-immunized and PCl-challenged mice that were treated with saline (*group C*). The positive 2-h and 24-h components of PCl CS responses (*group C*) were inhibited significantly in group D mice treated with sTNFR just before challenge, whereas treatment just after the 2-h early component, which could have no effect on the 2-h swelling, interestingly also did not affect the subsequent 24-h responses. This suggested that TNF-α functions early in CS initiation and, if not blocked during the early phase, proceeds to activate the vasculature to allow T cell recruitment. Thus, when sTNFR was given at 3 h, which is after activation of TNF-αR, there was no effect on 24-h elicited CS. *d*, RT-PCR was used to determine IFN-γ mRNA in CS ear extracts obtained at 4 h after local PCl challenge in previously PCl-sensitized (+) and in control vehicle-sensitized (−) mice. *e*, TCR-$\alpha^{-/-}$ and control BALB/c mice were contact sensitized as above and on day 4 were ear challenged with 0.8% PCl to elicit CS. Ear extracts were prepared 24 h after Ag challenge, and the level of IFN-γ was determined by ELISA.

challenge in separate groups of mice that previously were PCl-immunized or were sham-immunized with vehicle. At all times after Ag challenge, i.e., from 1 h through 24 h, chemotactic activity was elaborated into PCl-challenged CS ears of PCl-immunized mice and was dependent on immunization and not just on challenge. Thus, at each time point, chemotactic activity in ear extracts of immunized mice (Fig. 2, ■) was greater than in those of control vehicle-immunized mice (Fig. 2, □) when both were challenged equally. Furthermore, in another experiment, 24-h ear extract chemotactic activity again was significantly greater in PCl-immunized and PCl-challenged mice, compared with similarly challenged vehicle-immunized controls (Fig. 2, *group K* vs *group L*) and, PCl-challenged totally naive mice (*groups M* and *N*), and naive and unchallenged mice (*group O*). Taken together, these results suggest that by 4 days after contact sensitization, a particular aspect of

acquired immunity was induced, such that subsequent local Ag challenge on the ears triggered production of this very early local chemotactic activity, which was not elaborated by similar challenge of control vehicle-immune or naive mice.

Role of C5 and TCR in early phase chemotactic activity

To further study this early CS-initiating phase, we chose to examine the 2-h time point after Ag challenge, which is before arrival and activation of T cells (Fig. 1, *a*, *b*, and *e*), and compared it to the 24-h time point, which instead reflected Th1 cell-dependent aspects of CS. Twenty-four-hour chemotactic activity was reduced strongly in two C5-deficient strains, DBA/2 (Fig. 3*b*) and B10.D2/o (Fig. 3*c*). These strains were challenged on the ears with low doses of 0.4% PCl to more effectively demonstrate differences in CS of C-deficient strains, as described previously (18). This

FIGURE 2. Time course analysis of macrophage chemotactic activity in CS ear extracts. At day 4 after PCl contact sensitization and at various times after topical PCl Ag challenge on the ears, ear extracts from paired groups of immune (5% PCl-painted; ■) or nonimmune (vehicle-painted; □) CBA/J mice were assayed for chemotactic activity (*groups A and B*, 1 h; *groups C and D*, 2 h; *groups E and F*, 4 h; *groups G and H*, 8 h; and *groups I and J*, 24 h). Human C5a (100 ng/ml; *group Q*) and RPMI-gelatin medium (*group P*) were used for positive and negative controls, respectively. "Naïve" (*group O*) denotes chemotactic activity in ear extracts from naïve mice that were neither immunized nor challenged. Mice in *group N* were naïve mice that were ear challenged with 0.8% PCl. The number of migrated J774A.1 macrophages was determined.

Time Course Analysis of Ear Extract Chemotactic Activity in CS
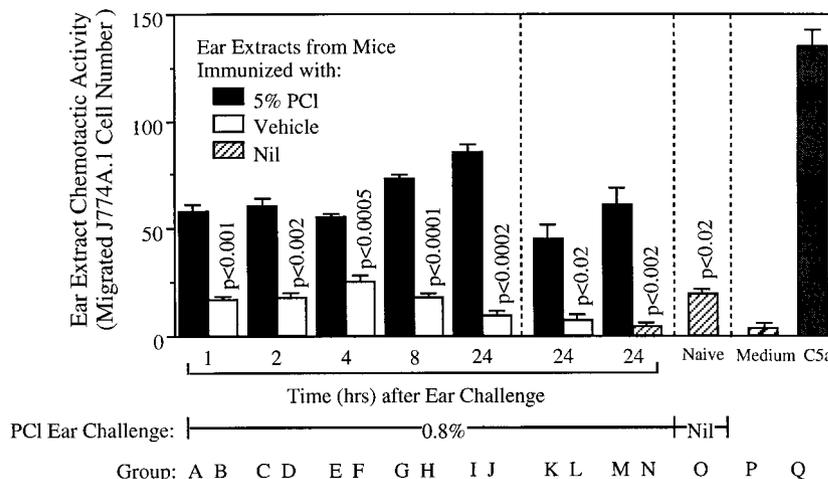
finding of decreased 24-h chemotactic activity in CS responses of C5-deficient mice was consistent with our prior findings that anti-C treatment decreased 24-h chemotactic activity (19). Importantly, elaboration of early chemotactic activity at 2 h also was dependent on C, because 2-h CS ear chemotactic activity was strongly reduced in C5-deficient DBA/2 mice compared with C5-normal DBA/1 mice (Fig. 3*a*). Therefore, both early and late CS chemotactic activity appeared to be dependent on C5. In contrast, TCRα$^{-/-}$ as well as TCRδ$^{-/-}$ mice had normal elaboration of early 2-h chemotactic activity in CS responses (Fig. 4*a* and *b*, *left panel*). As expected, αTCR$^{-/-}$ mice had no late 24-h chemotactic activity because this should be dependent on Ag-specific αβ-T cells (Fig. 4*a*, *right panel*). In contrast, the late 24-h chemotactic response in CS ear extracts was intact in TCRδ$^{-/-}$ mice vs C57BL/6 controls (Fig. 4*b*, *right panel*). Thus, although immunization was strictly required to elaborate both early and late chemotactic activity in CS, only the late-phase chemotactic activity was αβ-T cell-dependent and notably the early phase was T cell-independent.

Importantly, both early and late chemotactic activities in CS ears were dependent on C5, indicating that contact immunization induced a type of immunity that allowed for elaboration of C5-de-

pendent early chemotactic activity, upon which the subsequent elaboration of late chemotactic activity depended, probably because early chemotactic activity was a marker of early processes required for initial recruitment of T cells. Furthermore, elaboration of early 2-h chemotactic activity was Ag-specific, because only PCl-sensitized and PCl-challenged, but not OX-challenged mice (Fig. 5, *upper left panel*), or OX-sensitized and OX-challenged, but not PCl-challenged mice (Fig. 5, *upper right panel*), had significant 2-h chemotactic activity in ear extracts. As expected, chemotactic activity in 24-h CS ears also was Ag-specific (Fig. 5, *lower panels*), because Ag-specific late-acting T cells likely were required. These findings confirmed that early elaborated chemotactic activity, like 2-h early ear swelling (38), was a result of Ag-specific acquired immunity that was not mediated by T cells.

C5a and chemokines, respectively, largely account for early and late generated chemotactic activity in CS ears

Further study showed that the 2-h chemotactic activity probably was a result of C5a, because anti-rat C5a serum (39) neutralized the chemotactic activity detected in CS ear extracts (Fig. 6*a*, *middle columns*). The anti-serum was validated by its ability to neutralize chemotaxis mediated by ZAMS, which is due to C5a (Fig.
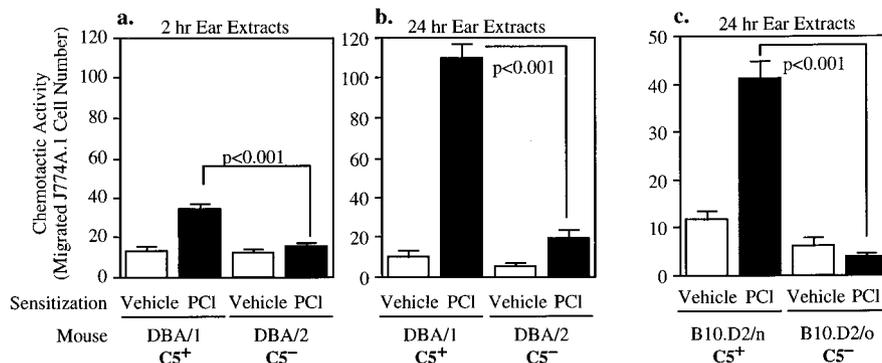
C5-Dependent Elaboration of Chemotactic Activity

FIGURE 3. Chemotactic activity in CS ear extracts of C5-deficient mice. *a* and *b*, DBA/1 and DBA/2 mice were contact sensitized with 5% PCl and ear challenged with 0.4% PCl on day 4. Two-hour (*a*) and 24-h (*b*) CS ear extract samples were assayed for chemotactic activity by determining the number of migrated J774A.1 cells. *c*, B10.D2/n and B10.D2/o mice were contact sensitized twice, on days 0 and 1, with 5% PCl and then ear challenged with 0.4% PCl on day 7. Twenty-four-hour extracts were assayed by determining number of migrated J774A.1 cells. The lesser 24-h chemotactic activity in C5^{+} B10.D2/n vs C5^{+} DBA/1 mice probably was associated with the lesser responsiveness for PCl-induced CS in C57BL/10 background mice.

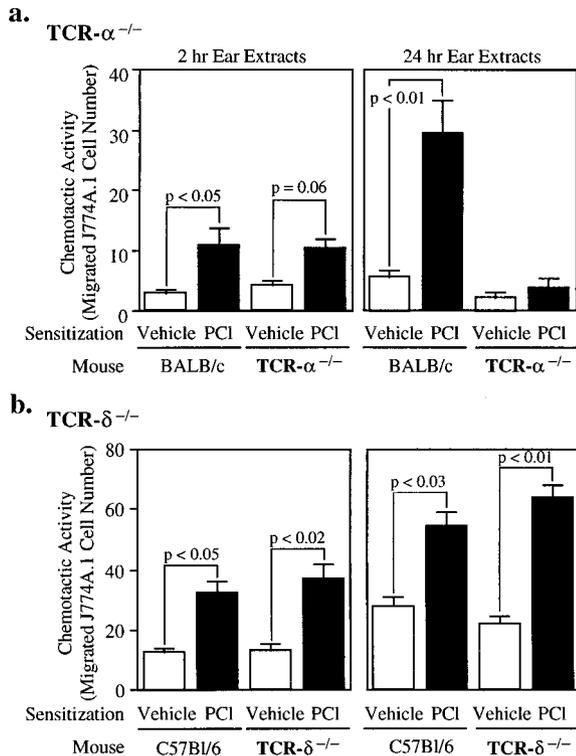## Chemotactic Activity in CS Ear Extracts of TCR α- and δ-Knock Out Mice

a. TCR-α $^{-/-}$

b. TCR-δ $^{-/-}$

FIGURE 4. Elaboration of chemotactic activity in CS ears from TCRα $^{-/-}$ and TCRδ $^{-/-}$ mice. *a*, TCRα $^{-/-}$ and normal BALB/c mice were contact sensitized with 5% PCl on day 0 and ear challenged on day 4 with 0.8% PCl. Ear extracts were prepared 2 and 24 h after challenge and assayed for chemotactic activity. *b*, TCRδ $^{-/-}$ and normal C57BL/6 mice were contact sensitized with 5% PCl on days 0 and 1 and ear challenged on day 7 with 0.8% PCl. Ear extracts were prepared 2 and 24 h after challenge and assayed for chemotactic activity.

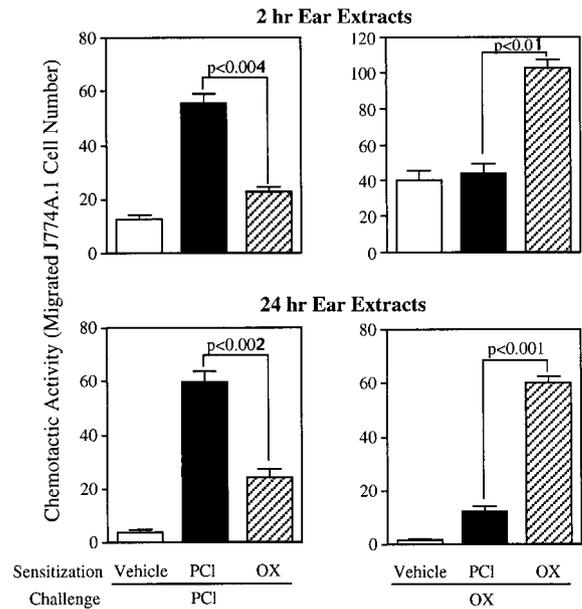## Ag-Specificity of Chemotactic Activity in CS Ear Extracts

FIGURE 5. Ag-specific elaboration of macrophage chemotactic activity in CS ears. CBA/J mice were sensitized with 5% PCl or 3% OX and then were challenged on the ears on day 4 with either by 0.4% PCl or OX. Two-hour and 24-h CS ear extract samples were assayed for J774A.1 chemotactic activity.

6*a*, *left columns*). In contrast, chemotactic activity in 24-h CS extracts was minimally inhibited by anti-C5a (Fig. 6*a*, *right columns*). We concluded that C5a was the dominant chemotactic factor present in early 2-h CS ear extracts, whereas other chemotaxins active on J774A.1 macrophages were involved in the elicitation of late 24-h CS.

We employed another specific method to verify the early participation of C5a by using macrophages from C5aR $^{-/-}$ mice (31) as targets in the chemotaxis assay to evaluate the presence of C5a in the CS ear extracts. As expected, C5aR $^{-/-}$ macrophages, compared with C5aR $^{+/+}$ macrophages, showed no chemotaxis against C5a contained in ZAMS (Fig. 6*b*, *right panel*). Importantly, chemotaxis of C5aR $^{-/-}$ macrophages against the 2-h CS ear extracts was impaired significantly, compared with C5aR $^{+/+}$ macrophages (Fig. 6*b*, *left panel*). In contrast, chemotaxis of the C5aR $^{-/-}$ macrophages against the 24-h ear extracts was not inhibited (Fig. 6*b*, *left panel*). We ascribed the nearly intact chemotactic activity of the late extracts on C5a $^{-/-}$ macrophages to the predominance of chemokines in the 24-h CS extracts (Fig. 7) that act on chemokine receptors rather than on C5aR. Again, as with anti-C5a (Fig. 6*a*), these findings indicated that C5a was dominant in early 2-h CS extracts and similarly showed that the chemotactic factors that predominated in late 24-h CS ear extracts were not C5a.

We postulated that chemokines could represent the other chemotaxins found at 24 h. Indeed, Fig. 7 shows that we detected Th1-associated MCP-1, MIP-1α, MIP-1β, and IP-10 in CS ear extracts at 24 h by specific ELISAs. These chemokines, like early C5a, also were elaborated in an immunization-dependent fashion at 2 h but were present in much greater amounts at 24 h after specific Ag challenge (Fig. 7). Thus, the four chemokines measured were detected in greater amounts in immunized mice than in nonimmunized mice, when both were equally PCl-challenged on the ears (Fig. 7). Because substantial amounts of chemokines were detected at 24 h, when C5a was found not to be responsible for chemotaxis (Fig. 6), the chemokines probably were responsible for the C5a-independent chemotactic activity noted in the 24-h CS ear extracts (Fig. 6). Overall, these results clearly indicated that C5a, which was generated via Ag-specific (Fig. 5) activation of C early (1–2 h; Fig. 2) after Ag challenge to elicit CS, accounted for early generated chemotactic activity. This early elaborated C5a likely participates in the initial recruitment of the T cells, which are then activated in the tissues starting at 4 h via Ag/MHC on APC to produce IFN-γ (Fig. 1). The even later elaboration of chemokines detected at 24 h (Fig. 7), which probably are largely derived from local tissue cells and are dependent on T cell cytokines like IFN-γ (40), would then lead to recruitment of nonspecific leukocytes that are found later at 24 h, such as monocytes.

Local early elaboration of C5a is crucial for later elicitation of 24 h CS

Use of C5aR $^{-/-}$ mice gave us the opportunity to determine the absolute requirement for C5a and C5aR in CS responses. Measurement of early 2-h and late 24-h chemotactic activity in extracts of CS ears from C5aR $^{-/-}$ mice produced an interesting result. The immunized and PCl-challenged C5aR $^{-/-}$ mice had greatly augmented C5a-dependent early 2-h chemotactic activity in CS ear extracts, compared with that of C5aR $^{+/+}$ mice (Fig. 8*a*). This striking finding may have been because of absent clearance of C5a elaborated locally early in CS, which may depend on binding to the

a. Effects of C5a-Neutralization *in vitro* via Antiserum on Chemotactic Activity in Ear Extracts
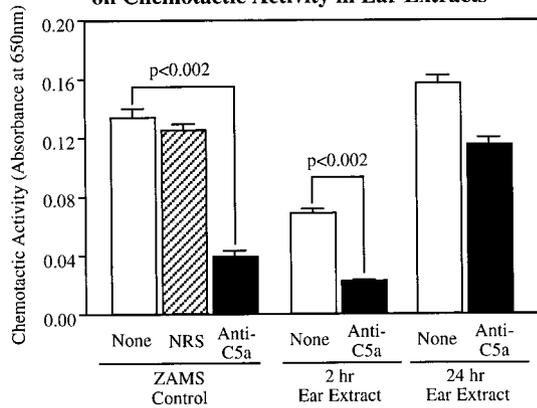
b. Chemotaxis of C5aR-deficient Macrophages to CS Ear Extracts
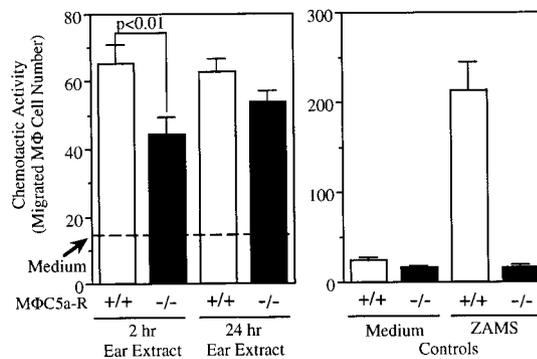

FIGURE 6. *a*, Inhibition of chemotactic activity in 2-h CS ear extracts by neutralizing C5a antiserum. Two-hour and 24-h ear extracts and ZAMS, as a murine C5a chemotactic control, were preincubated with rabbit anti-rat C5a serum or with normal rabbit serum and then were assayed for chemotaxis. In this experiment, extraction of dye used to stain migrated J774.A1 cells on filter spots was performed, and absorbance at 650 nm was determined. *b*, Impaired chemotaxis of C5aR$^{-/-}$ peritoneal exudate macrophages (PEC) against 2-h ear extracts. PEC were prepared from C5aR$^{+/+}$ and C5aR$^{-/-}$ mice injected i.p. with 3 ml thioglycolate 4 days before. Chemotaxis assay was performed similarly to the assay for J774A.1 cells, and number of migrated PEC was determined.
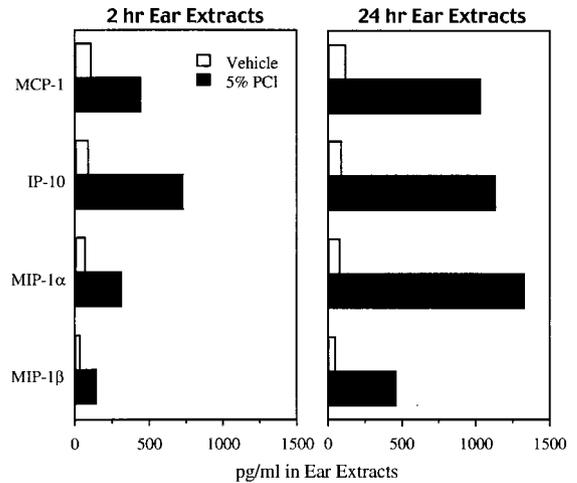
Quantitation of Chemokines in CS Ear Extracts

FIGURE 7. Chemokine elaboration in CS ear extracts. CBA/J mice were contact sensitized with 5% PCl and ear challenged with PCl on day 4. Two-hour and 24-h CS ear extract samples were assayed for chemokines (MCP-1, IP-10, MIP-1α, and MIP-1β) by specific ELISA.

highly significant augmented 2-h and also mildly significant augmented 24-h ear swelling responses in actively PCl-sensitized mice treated with Plummer's reagent (Fig. 9). These findings strongly suggested that C5a was generated early after Ag challenge. Furthermore, it is noteworthy that the data in Fig. 9, *group D*, show that prolongation of early C5a activity in vivo actually enhanced the 24-h T cell-dependent late aspect of CS.

Taken together, these results suggest that the early recruitment of Th1 cells into the specific Ag-challenged local skin sites of CS elicitation requires early Ag-specific local generation of C5a, via C and C5 activation to generate C5a. Local early generated of C5a in CS ears may then interact with C5aR on several local candidate target cells, which then facilitate the migration of T cells across the vessels and into the extravascular tissue spaces. Migrated T cells are then activated by specific Ag/MHC complexes expressed on local APC to subsequently produce cytokines that mediate the late classical aspects of CS.

Discussion

Summary

We studied how Ag-specific effector T cells are recruited into Ag-challenged sites during elicitation of CS in previously sensitized mice. We focused on the early CS-initiating phase that precedes and leads to T cell recruitment into the local extravascular tissue site to mediate the classical late delayed phase of CS responses. We established that C was activated in this CS-initiating phase, leading to early local generation of C5a, which we demonstrated directly in early CS ear extracts. Elaboration of C5a was because of an Ag-specific process, and it occurred as early as 1 h after Ag challenge and required prior immunization, but unlike the late phase of CS, it was not dependent on $\alpha\beta$-T cells. The data suggest that early Ag-specific generation of C5a is required for elicitation of CS, by mediating initial T cell recruitment into the local Ag-challenged site rather than the elaborated C5a acting predominantly in the late phase of CS, e.g., to recruit monocytes.

Use of IFN-γ to differentiate CS initiation at 2 h from T cell-dependent late aspects of CS

We used the term "CS initiation" to signify the early processes required for recruitment of T cells into the tissues in CS. To differentiate

C5aR, which are absent in these mice. Thus, the absence of C5aR may have led to the enhanced local levels of C5a that were just found in the 2-h CS ear extracts. This finding lends further support to the idea that C5a is elaborated early after Ag challenge in CS reactions. On the other hand, chemokine-mediated chemotactic activity of late 24-h CS ear extracts was greatly reduced in C5aR$^{-/-}$ mice (Fig. 8*b*, *right panel*). This finding suggested that impaired T cell recruitment that occurred in CS responses of C5aR$^{-/-}$ mice likely was a result of the absence of an early-phase C5a interaction with C5aR. Accordingly, this lack of T cell recruitment led to impaired cytokine production and reduced expression of chemokines at 24 h (Fig. 8*b*, *right panel*). Indeed, C5aR$^{-/-}$ mice had severely impaired elicitation of 24-h CS after PCl sensitization and ear challenge on day 4, compared with that of C5aR$^{+/+}$ mice (Fig. 8*c*).

We employed yet another, independent method to evaluate a role for C5a in elicitation of CS by treating mice with Plummer's reagent. This substance inhibits carboxypeptidase N, the C5a-metabolizing enzyme that converts C5a to inactive C5a des arg, resulting in increase and prolongation of intact C5a (41). We found

a. **2 hr Chemotactic Activity in CS Ear Extracts from C5a-R$^{-/-}$ Mice**

c. **24 hr CS in C5a-R$^{-/-}$ Mice vs. Controls**

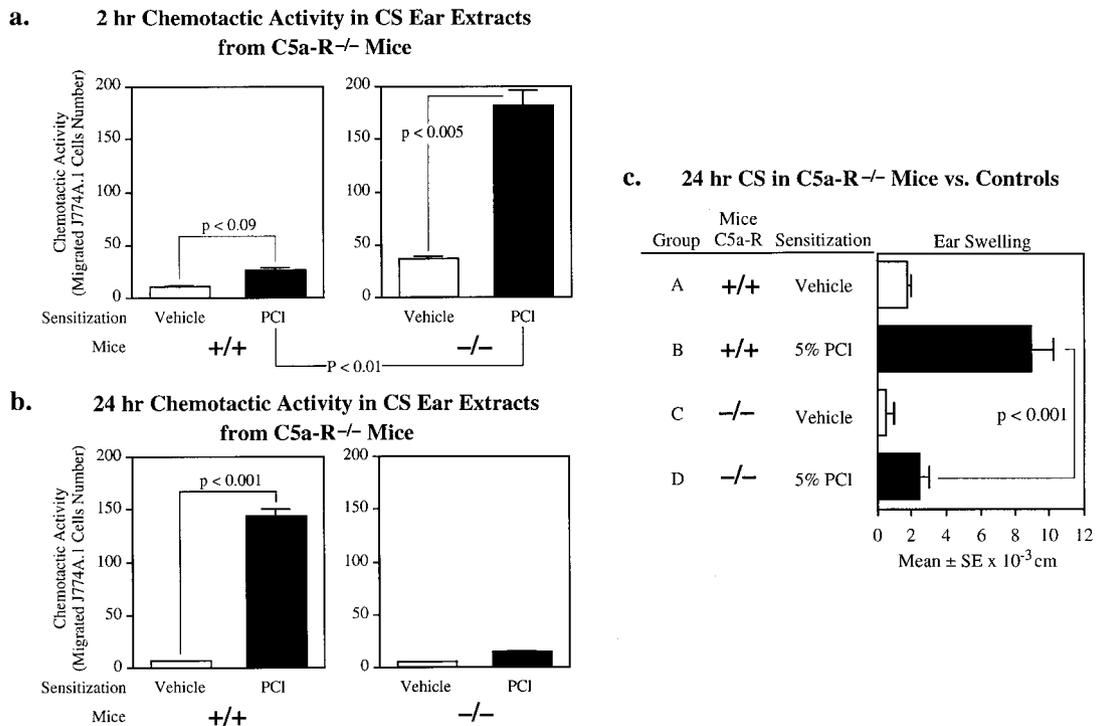**b.** **24 hr Chemotactic Activity in CS Ear Extracts from C5a-R$^{-/-}$ Mice**

FIGURE 8. Early involvement of C5a in elicitation of CS responses. C5aR$^{+/+}$ and C5aR$^{-/-}$ mice were contact sensitized twice, on days 0 and 1, with 5% PCl and then ear challenged with 0.8% PCl on day 7. Two-hour (*a*) and 24-h (*b*) CS ear extract samples were assayed for chemotactic activity. *c*, Resulting 24-h CS ear swelling responses also were measured.

early CS initiation from the later T cell-dependent inflammatory phase, we used IFN-γ as an in situ marker for the end of the early phase and beginning of the late phase, thus denoting the arrival and subsequent Ag activation of recruited T cells into locally challenged sites. Because Th1 and Tc1 subsets can mediate CS and DTH by selectively producing IFN-γ in response to local Ag/MHC stimulation by APC in the tissues (1–3, 36, 42), it was reasonable to measure IFN-γ as a marker of the 24-h late phase. However, it was noteworthy that IFN-γ mRNA and protein first could be detected locally as early as 4 h after Ag challenge (Fig. 1, *a*, *b*, and *d*). In addition, we showed that TNF-α, which likely leads to IFN-γ, acted before 3 h (Fig. 1*d*). This fits with TNF-α-dependent expression of VCAM-1 and ICAM-1 on the luminal surface of local endothelial cells in CS by 4 h after challenge (17). This likely enables the first T cells to undergo trans-endothelial migration, because direct injection of TNF-α induces expression of vascular adhesion molecules in 2 h (17). In addition,

IFN-γ was not found at 4 or 24 h in CS ears of TCR$^{-/-}$ mice (Fig. 1, *d* and *e*), confirming the usefulness of measuring IFN-γ in evaluation of αβ-T cell recruitment. Together these findings assured that analysis of biochemical events in ear extracts at just 1–2 h after Ag challenge was a manifestation of CS-initiating events that precede and are required for subsequent recruitment of the CS-effector T cells to then be activated by APC to produce IFN-γ locally.

Chemotactic activity induced early (1–2 h) after Ag challenge of CS ears was a marker of CS initiation

We previously used J774.A1 macrophages as indicator cells to show that chemotactic activity was a good molecular marker to evaluate the 24-h aspect of CS responses (19). Also, we showed that C5a selectivity among C components mediated chemotaxis of J774A.1 cells, which lacked chemotaxis against ZAMS without C5a, which had been prepared from C5-deficient mouse sera (our

Effect of Plummer's Reagent, an Inhibitor of C5a-Catabolism, on CS responses
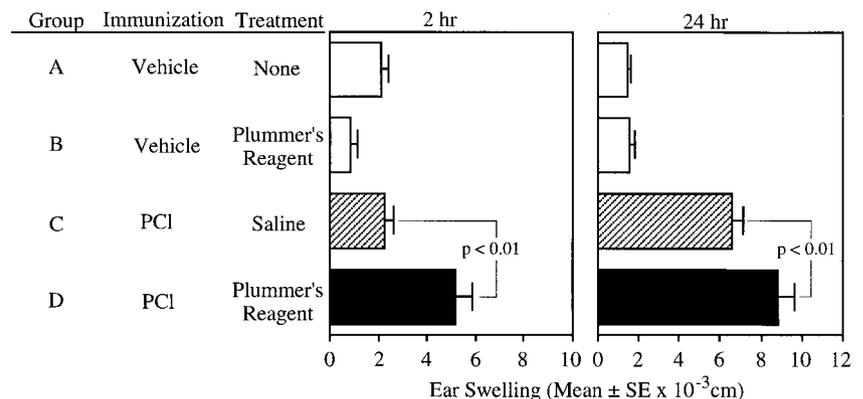
FIGURE 9. Prolongation of C5a activity by administration of Plummer's reagent led to augmented CS. CBA/J mice were contact sensitized on day 0 with 5% PCl and then ear challenged with 0.8% PCl on day 4. Ear swelling was measured at 2 h and 24 h after Ag challenge. Plummer's reagent was injected i.p. at a dose of 100 mg/kg 30 min before local Ag challenge on the ears.

unpublished result). Thus, our prior findings of decreased chemotactic activity in 24-h CS ears after anti-C treatments, such as anti-C5 mAb or sCR1 (19), suggested that C5a might be responsible for the chemotactic activity in CS ear extracts. However, in those prior studies of 24-h chemotactic activity, there was neither an exact identification of C5a nor a determination of the point at which various potential chemotaxins acted in the separate early and late phases that are needed for elicitation of CS. In contrast, the experiments of the current study identified that C5a was present early and not late and determined the possible role of C5a in early occurring CS initiation. We first performed a time course analysis of chemotactic activity in CS ears after Ag challenge of contact-sensitized mice and found that chemotactic activity was induced as early as 1 h after Ag challenge of sensitized mice (Fig. 2). We examined the C-dependency of early 2-h vs late 24-h chemotactic activity in CS ears of C5-deficient mice and found that elaboration of both the early and the late chemotactic activity was C5-dependent (Fig. 3). Interestingly, early 2-h chemotactic activity was elaborated normally in $\alpha\beta$-T cell- and $\gamma\delta$-T cell-deficient mice (Fig. 4), but the late 24-h chemotactic activity required the presence and activation of $\alpha\beta$-T cells (Fig. 4a). These findings suggested that early chemotactic activity was not dependent on T cells, but that late chemotaxis was quite different and was dependent on $\alpha\beta$-T cells, which probably depended on early C5a for local recruitment in CS.

C5a and chemokines, respectively, were largely responsible for 2-h and 24-h chemotactic activity

We employed Ab neutralization of C5a activity (Fig. 6a) and C5aR-deficient macrophages as migrating indicator cells in vitro (Fig. 6b) to confirm that the chemotactic activity detected early in CS ears was due to C5a. Although early 2-h chemotactic activity was diminished employing both methods, late 24-h chemotactic activity was not affected by either method, suggesting that C5a was mostly responsible for early 2-h chemotactic activity and that other chemotaxins were involved in the late 24-h CS ear chemotactic activity, which required C5a recruitment of $\alpha\beta$-T cells in order to be elaborated. These findings suggesting that C5a acted early are in accord with our previous observation that inhibition of CS with sCR1 required that this C inhibitor be given before Ag challenge, whereas anti-C treatment given just 3 h after Ag challenge was no longer effective (19). In addition, early 2-h C5a chemotactic activity was greatly augmented in C5aR-deficient mice (Fig. 8a, *right panel*), suggesting that C5a was elaborated early, and in this instance was not adsorbed out from tissues, perhaps because of the absence of binding to C5aR. Finally, neither late 24-h chemotactic activity (Fig. 8b) nor late 24-h ear swelling (Fig. 8c) was elicited in C5aR$^{-/-}$ mice, confirming the crucial role of early interactions between C5a and C5aR in early CS-initiating steps leading to T cell recruitment for elicitation of late chemokine elaboration and macroscopic CS responses.

The importance of chemokines in immune inflammation recently has been described (43), and some chemokines were reported to be mediators in CS responses (44). Because J774A.1 cells also can respond to some chemokines (our unpublished results), we measured chemokines in CS ear extracts by specific ELISA. We found that MIP-1α, MIP-1β, MCP-1, and IP-10 also were produced in 2-h CS ears, but they were present in much greater amounts in the 24-h CS ear extracts (Fig. 7). Importantly, like C5a (Fig. 2), elaboration of these chemokines was not induced by the irritating effect of the hapten Ag (PCl) used to challenge the ears because large amounts of the chemokines only were detected in mice that were both previously immunized and then ear challenged (Fig. 7). Therefore, these results suggested that late 24-h

chemotactic activity in CS principally was due to local elaboration of chemokines. Thus, we concluded that sCR1 and anti-C5 inhibition of early activation of C and of C5 needed to generate C5a led to impaired T cell recruitment, causing reduced chemokines found in late 24-h CS ear extracts, because elaboration of late 24-h chemotactic activity was dependent on $\alpha\beta$-T cells (Fig. 4a). Furthermore, the chemokines detected in CS fit with the type of CS-effector T cell in that they were relatively Th1-associated, particularly IP-10 (44, 45), which is induced by IFN-γ in local tissue cells, particularly in the skin by keratinocytes (40).

Significance of C5a in CS

The requirement for C5a in elicitation of CS was clearly demonstrated by the strongly impaired 24-h CS responses and 24-h chemotactic activity in C5aR$^{-/-}$ mice (Fig. 8). Also, administration of Plummer's reagent, which causes enhanced and prolonged C5a activity by inhibiting the C5a-inactivating enzyme, carboxypeptidase N, and thus leads to inhibited generation of inactive C5a-desArg (41), resulted in significant augmentation of both early 2-h and late 24-h ear swelling responses (Fig. 9). Thus, augmentation of local C5a levels by Plummer's reagent led to augmented elicitation of both early 2-h and late 24-h CS. Taken together, these genetic and biochemical findings make it likely that C5a, which is elaborated very early after Ag challenge (Fig. 2), plays a crucial role in CS by leading eventually to the migration of effector Th1 cells into extravascular sites of CS elicitation.

It is important to note that early 2-h chemotactic activity, which likely was because of C5a, was elaborated Ag-specifically (Fig. 5) but independent of T cells (Fig. 4). This Ag specificity of C-activation to generate local elaboration of C5a, together with its immunization-dependency (Fig. 2) and lack of T cell-dependence (Fig. 4), strongly suggested that Ab derived from B cells might be involved in the early CS-initiating cascade. In fact, we previously demonstrated that CS was impaired in B cell-deficient μMT mice (19). Further data we present in another paper suggest that early after sensitization, the B-1 cell subset of B cells in central tissues produces circulating IgM Ab to mediate local Ag-specific C activation and to generate C5a locally (R. F. Tsuji, M. Szcepanik, I. Kawikova, R. Campos, M. Akahira-Azuma, and P. W. Askenase, manuscript in preparation).

Overall scheme for elicitation of CS

Our studies have established that early elaboration of C5a is required for Th1 cell recruitment in CS. An important question concerns how C5a actually leads to T cell recruitment. Does C5a act directly as a chemotactic factor for attraction of the CS-effector T cells into the tissues, or does C5a act indirectly via triggering C5aR on local mediator cells to release substances that influence the tissue microenvironment to facilitate local T cell recruitment? Recently, it was shown that C5a is chemotactic in vitro for activated human T cells and that C5aR are expressed in vivo on T cells in DTH-related encephalomyelitis in rats (23). This suggests that C5a may act directly to recruit recently immunized T cells. Alternatively, although we assayed C5a in vitro as a chemotactic factor for macrophages and this also might apply to T cells, it also is likely for two reasons that C5a indirectly leads to T cell recruitment in CS by influencing the local microenvironment in CS. First, local mast cells and platelets, which are established to express functional C5aR (28–30), were shown previously to be involved in CS initiation by local release of 5-HT (10–13) and TNF-α (17), which activate the endothelium to facilitate local T cell recruitment across the vessels (17, 22). In the current study, we confirmed the early involvement of TNF-α (Fig. 1d). Second, C5a also can activate endothelium directly through endothelial cell C5aR (46, 47).

Taken together, we propose, in addition to a possible direct attraction of T cells by C5a, that T cell recruitment in CS requires C5a-dependent release of TNF-α and 5-HT, which are produced in the early CS-initiating phase by local C5aR-bearing mediator cells, like mast cells and platelets, and/or that C5a acts directly to facilitate local T cell recruitment by activating C5aR on the endothelial cells. Activation of endothelium for permeability and expression of adhesion molecules (17) allows T cells to bind to and then migrate across the endothelium into the tissues.

After extravasation of the recruited T cells into the tissues, C5a may also facilitate T cell chemotaxis directly and perhaps may contribute to T cell movement toward conjugation with local APC. In the tissues, the subset of the recruited T cells that are specific for the challenging Ag are then selected to bind with specific Ag/MHC complexes on the local APC, causing T cell activation and release of IFN-γ to mark the onset of the late phase and the end of the early CS initiation phase. Then eventually, T cell cytokines like IFN-γ, produced by the Ag/MHC-activated T cells, generate chemokines by activation of local tissue cells (40, 44, 45). The chemokines, rather than C5a, most likely are required for later late-phase monocyte recruitment that is associated with elicitation of 24-h CS ear swelling because tissue monocytes and ear extract chemokine levels both are increased in CS ear tissues in the later phase of CS responses, whereas C5a was decreased in CS ear extracts in this later phase. Thus, although we employed in vitro monocyte chemotaxis as an assay method to detect C5a in CS ear extracts, our data suggest that C5a does not act late in CS as a dominant chemotaxin for monocytes, but is elaborated very early in the initiation phase of CS responses and then acts early in elicitation of CS to lead to initial T cell recruitment. In contrast, we found that monocyte recruitment in CS was more remotely dependent on C5a because C5a acted indirectly via early attraction of T cells and, after local Ag/MHC activation of the T cells, led to monocyte recruitment, predominantly via T cell-dependent chemokines released from tissues cells.

In summary, our present results suggest that early phase generation of C5a via Ag-specific C activation is required for important and underinvestigated crucial initiating aspects of CS effector T cell responses in vivo. These components of CS initiation were elicited by ear challenge as soon as day 4 after immunization and occur quite early (within 1–2 h) after elicitation by local challenge with Ag. Besides mediating possible T cell chemotaxis, C5a probably triggers a local CS-initiating cascade in the tissues. Accordingly, C5a likely acts on mast cell and platelet C5aR, leading to release of mediators, particularly TNF-α and also 5-HT, that activate local endothelia to facilitate T cell recruitment. In contrast, C5a does not appear to act dominantly in the late phase of CS to attract monocytes, which is more likely due to T cell-dependent chemokines. Understanding these newly delineated initiating aspects of T cell immunity in vivo may be critical for development of new therapeutic strategies to control clinically important T cell-mediated disease processes by regulating these crucial early steps.

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