Self heat shock and $\gamma\delta$ T-cell reactivity

(mycobacterial antigens/stress proteins/ $\gamma\delta$ genes)

RAMANUJAM RAJASEKAR*, GEK-KEE SIM†, AND ANDREI AUGUSTIN*‡

*Department of Medicine, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO 80206; ‡Department of Microbiology and Immunology, University of Colorado Health Sciences Center, Denver, CO 80262; and †Basel Institute for Immunology, Grenzacherstrasse 487, CH-4005 Basel, Switzerland

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ABSTRACT We have investigated the effects of heat shock on T-cell induction and selection *in vitro*. We find that when cell preparations containing T lymphocytes are incubated for 30 min at 42°C, a selective proliferation of $\gamma\delta^+$ T cells bearing the $\gamma\delta$ T-cell antigen receptor follows. A greater enrichment of $\gamma\delta^+$ T cells is observed, upon preexposure to mycobacterial antigens *in vivo*. By comparing the effects of heat shock with that of mitogen or specific T-cell triggering by conventional antigens and by analyzing the $\gamma\delta$ T-cell receptor genes expressed in cells that proliferate as a result of heat shock induction, we conclude that a subset of murine $\gamma\delta$ T cells react to antigens on self cells in which a heat shock response was induced.

The immune reactivity to stress proteins is currently under intense scrutiny. Extensive regions of stress proteins have been shown to be well conserved in phylogeny from prokaryotes to eukaryotes and between the plant and animal kingdom (1, 2). The immunodominant mycobacteria protein antigens responsible for the reactivity against various infections in mice and man are actually homologs of stress proteins (1). The relationship, if any, between immune reactivity to stress proteins and to mycobacterial infections, however, is unclear.

The T-lymphocyte antigen receptors (TCRs) on the majority of T cells in human and mice are of the $\alpha\beta$ type (TCR $\alpha\beta$). A second type of TCR, the $\gamma\delta$ receptor (TCR $\gamma\delta$), has been described only recently. The biological function of the $\gamma\delta$ T cells has yet to be established. Although most T lymphocytes specific for mycobacterial antigens appear to be CD4⁺ and express the TCR $\alpha\beta$ (1, 3, 4), there are reports that murine and human T cells that carry the TCR $\gamma\delta$ react to a 65-kDa mycobacterial heat shock protein hsp65 (5–8). Moreover, murine $\gamma\delta$ T cells from lymph nodes (9) and lungs (10) can be preferentially triggered *in vivo*, as a consequence of local exposure to *Mycobacterium tuberculosis* antigens, among which stress proteins are well represented. One could propose that T-cell-defined epitopes are contained in those regions of stress proteins that are evolutionarily conserved and that, since stress proteins are overexpressed *in vivo* only under specific circumstances, T-cell reactivity against these self proteins could be demonstrated in adult animals. Thus, the recognition of both bacterial and self stress proteins by T cells may contribute to immune reactions at various sites of pathogen infection. To test this view, we investigated the activation and responsiveness of T lymphocytes under conditions of stress.

A simple way of eliciting stress-induced protein synthesis is by exposure to elevated temperatures. In preliminary trials, we have determined that a brief exposure at 42°C is sufficient for inducing heat shock stress protein synthesis in a lymphoid population but does not appreciably affect the

proliferation of T cells. Subsequently, we have designed an experimental protocol that could reveal the effects of heat shock on T-cell induction and selection *in vitro*. Under our experimental conditions, we detected a preferential proliferation of $\gamma\delta^+$ T lymphocytes, compared to the $\alpha\beta^+$ T cells. The enrichment of $\gamma\delta^+$ T cells was particularly marked upon exposure first to mycobacteria antigens *in vivo* and then to heat shock *in vitro*. In such T-cell populations, the analysis of γ and δ variable (V) and joining (J) gene segments utilized indicates that a heterogenous but well-defined set of $\gamma\delta$ receptors is expressed.

MATERIALS AND METHODS

Animals. BALB/c mice (6–8 weeks old) were purchased from the Jackson Laboratory.

Immunization. Resident pulmonary lymphocytes (RPLs) immunization by aerosols of purified protein derivative (PPD) was performed as described (10). Immunized lymph nodes were periaortic and inguinal lymph nodes of mice injected at the base of the tail (11) with either complete Freund's adjuvant (CFA; 100 μ l per mouse; GIBCO), incomplete Freund's adjuvant (100 μ l per mouse; Difco) or CFA plus antigens [keyhole limpet hemocynin (KLH; Calbiochem) or ovalbumin (OVA; Sigma)] at 100 μ g per mouse.

Heat Shock. Temperature and duration of heat shock were established by several trials in which the survival and proliferative potential of all T cells were determined. In general, heat shock was performed prior to the initiation of culture. Cells were resuspended at a concentration of 10⁶ cells per ml in medium and incubated in a Fisher Versa-therm water bath set at 42°C $\pm$ 0.2°C for 30 min. Control samples were incubated in parallel in a bath set at 37°C. For [³⁵S]methionine labeling, cells were resuspended in methionine-free RPMI 1640 medium supplemented with 10% (vol/vol) dialyzed fetal calf serum during the incubation at 42°C. Subsequently, [³⁵S]methionine (Amersham) at 1 mCi/ml (1 Ci = 37 GBq) was added to the medium and the cells were cultured in a 5% CO₂/95% air incubator for a further 6 hr.

Cell Cultures. Lymphocyte suspensions from lymph nodes were prepared by standard procedures, and RPL populations were prepared as described (10). Cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum essentially as described (10, 11). Where applicable, lymphokines added after 3 days in culture were recombinant interleukin 1 and recombinant interleukin 2 (10 units/ml and 40 units/ml, respectively; gifts of P. Lomedico, Roche, Nutley, NJ). In general, 5 days after the addition of lymphokines, viable cells were analyzed. Where indicated,

Abbreviations: TCR, T-cell receptor; RPL, resident pulmonary lymphocyte; PCR, polymerase chain reaction; CFA, complete Freund's adjuvant; PPD, purified protein derivative; OVA, ovalbumin; mAb, monoclonal antibody; V, variable; C, constant; J, joining; C γ , γ chain C region; J δ , δ chain J region; V δ , δ chain V region; etc.; Con A, concanavalin A.

OVA and KLH were added at 40 $\mu\text{g}/\text{ml}$ and concanavalin A (Con A) at 5 $\mu\text{g}/\text{ml}$.

Immunofluorescence and Cell Cycle Analysis of $\gamma\delta^+$ T Cells. Viable cells were separated from the dead cells by Ficoll/hypaque centrifugation, and 2×10^5 cells were incubated with 50 μl of biotin-conjugated monoclonal antibody (mAb) anti- $\alpha\beta$ TCR (mAb H57-597) at 20 $\mu\text{g}/\text{ml}$ at 4°C for 30 min, washed, and incubated with the same amount of fluorescence conjugated anti-CD3 (mAb 145-2C11) plus streptavidin-phycoerythrin (Tago) for another 30 min at 4°C, washed, and analyzed on a Coulter Epics-C flow cytometer as described (10). $\gamma\delta^+$ T cells were purified by fluorescence-activated cell sorting as $\text{CD}3^+ \alpha\beta^-$ cells from a heterogeneous population containing both $\alpha\beta^+$ and $\alpha\beta^-$ T cells, by using an Epics-751 flow cytometer. The sorted cells were washed and incubated in 0.1% sodium citrate containing propidium iodide (50 $\mu\text{g}/\text{ml}$; Sigma) and DNase-free RNase (200 $\mu\text{g}/\text{ml}$; Boehringer Mannheim). The fluorescence intensity of propidium iodide-stained nuclei was measured in the same Epics flow cytometer. The number of cells in the various cell cycle phases was calculated by integrating the area under defined curve segments with the Epics software.

V Gene Segment Usage Analysis. RNA was prepared by the APGC procedure (12) from cell populations that were depleted of $\alpha\beta^+$ T cells by treatment with biotin-conjugated mAb H57-597 and streptavidin-coupled Dynal beads in conjunction with a Dynal magnetic particle concentrator. cDNA synthesis and polymerase chain reactions (PCRs) were performed accordingly (13). RNA from 10^5 cells was primed either with 10 pmol of γ chain C region (C_γ) primers (GGGAAATGTCTGCATCAAGC) or δ chain C region (C_δ) primers (AACAGATGGTTTGGCCGGAG) in 20- μl reaction mixtures for reverse transcription. Two percent of the cDNA synthesized was used in each PCR. Both 5' and 3' primers were present at 0.5 μM . The PCR was performed on a Cetus DNA Thermal Cycler as follows: 94°C for 30 sec, 52°C for 30 sec 72°C for 2 min for a total of 25 cycles, followed by a 6-min incubation at 72°C. All primers used were standardized with cloned DNA with the exception of $V_{\delta 3}$. The 3' primer for the γ PCR is above C_γ primer, and J $\delta 1$ (TTCCACAGTCACT-TGGGTTC) and J $\delta 2$ (CTCCACAAAGAGCTCTATGC) were used for the δ PCR. The 5' V primers are as follows: $V_{\gamma 2}$ (TTATCGGTCACCAGAGAGAC); $V_{\gamma 4}$ (ACCAAGAGAT-GAGACTGC); $V_{\gamma 5}$ (TAAGTTGCAAGCTCTCTGGG); $V_{\gamma 6}$ (GAAGCCCGATGCATACATAC); $V_{\gamma 7}$ (GATACTCACA-GAACAGGCAC) (Nomenclature for V_γ is from ref. 14.); $V_{\delta 1}$ (GGGATCCTGCCTCCTTCTAC); $V_{\delta 3}$, $V_{\delta 4}$, and $V_{\delta 7}$ primers are as in ref. 15; and $V_{\delta 6}$ (AGGTCCAGTCAACAG-GCAGC). All primers are listed 5'-3'. Sequences are derived from refs. 16–22.

RESULTS

A short exposure to high temperature (heat shock) does not impair the proliferative potential of T cells while inducing the *de novo* synthesis of significant amounts of heat shock proteins in lymphocyte populations (Figs. 1 and 2). In Fig. 1, we have used the murine RPL population (10) to show that a 30-min incubation at 42°C is sufficient for the induction of characteristic heat shock protein synthesis. Accordingly, we have devised a simple procedure for detecting the effect of heat shock on T-cell induction and selection *in vitro*. Briefly, lymphocytes are isolated from organs of normal or topically primed BALB/c mice and incubated at 42°C for 30 min. Subsequently, cells are cultured *in vitro* for 3 days in the absence of any added lymphokines. Assuming that T cells that can react to heat shock-induced antigens should have been activated and display lymphokine receptors, a mixture of interleukins 1 and 2 was then added and the T-cell population was expanded for 5 additional days before anal-

FIG. 1. Major heat shock proteins induced in a RPL population by treatment at 42°C for 30 min. Heat shock and control cells (10^6 cells per ml) were labeled with [^{35}S]methionine at 1 mCi/ml at 37°C for 6 hr. Labeled proteins were extracted with 0.2% Triton X-100 and 2×10^5 cpm were loaded in each lane and analyzed by SDS/PAGE on a 10% polyacrylamide gel. Lanes: A, non-heat-shocked RPLs; B, heat-shocked RPLs. Molecular mass standards are in kDa. Arrows indicate the major heat shock proteins induced.

ysis. The proportion of $\text{CD}3^+ \text{TCR}\alpha\beta^- / \text{CD}3^+ \text{TCR}\alpha\beta^+$ T cells in the selected population was determined by immunofluorescence. These $\text{CD}3^+ \text{TCR}\alpha\beta^-$ cells express the $\text{TCR}\gamma\delta$, as demonstrated by immunoprecipitation of the $\text{TCR}\gamma\delta$, performed as described (ref. 10 and data not shown) and by analysis of the mRNA of purified $\text{CD}3^+ \text{TCR}\alpha\beta^-$ T cells (see Fig. 4).

There are several experimental circumstances in which a substantial increase in the fraction of $\gamma\delta^+$ T cells is observed *in vitro* after heat shock. Lymphocytes that are confronted *in vivo* with an inactivated mycobacteria, *Mycobacterium smegmatis* from CFA, or PPD from *M. tuberculosis* display an increased proportion of $\gamma\delta^+$ T cells after heat shock and culture (Fig. 2). For example, cells from CFA-primed lymph nodes respond with an increase of the $\gamma\delta^+$ T cells from 5% in the non-heat-shocked control to 35% in the heat-shocked population. However, in cells isolated from unprimed lymph nodes or lymph nodes exposed to incomplete Freund's adjuvant, heat shock has little observable effect on the ratio between $\alpha\beta^+$ and $\gamma\delta^+$ T cells proliferating *in vitro*. This is intriguing since various conventional protein antigens have been administered *in vivo*, mixed with CFA, as a standard immunization procedure for obtaining antigen-specific T cell lines and hybrids expressing the $\text{TCR}\alpha\beta$ (11, 23, 24). In the same figure, we show that indeed, from the draining lymph nodes of mice subjected to such conventional immunizations (KLH or OVA plus CFA), $\gamma\delta^+$ T cells can be selectively expanded after heat shock. Alternatively, $\alpha\beta^+$ T cells can proliferate preferentially in non-heat-shocked populations if the priming antigen is continuously present in culture.

We have reported that in RPLs primed *in vivo* by exposing mice to aerosols containing PPD, $\gamma\delta^+$ T cells can constitute >50% of the proliferating $\text{CD}3^+$ T cells when propagated *in vitro* in the presence of PPD and lymphokines (10). In Fig. 2, RPLs isolated from PPD-primed murine lungs, when subjected to heat shock treatment, yield, after 8 days in culture, a T-cell population of which 65% are $\gamma\delta^+$. In contrast, in the non-heat-shocked lymphocytes of the same source subjected to identical culture conditions, only 22% of T cells are $\gamma\delta^+$. In normal unprimed RPLs, the effect of heat shock on the further enrichment of $\gamma\delta^+$ T cells *in vitro* is reproducibly measurable but less pronounced.

It is conceivable that $\gamma\delta^+$ T cells could be more resistant to heat shock than their $\alpha\beta^+$ counterpart. To estimate the viability and proliferative potential of $\alpha\beta^+$ lymphocytes after

FIG. 2. Preferential proliferation of $CD3^+ \alpha\beta^-$ T cells after heat shock. Approximately 2×10^5 viable cells from each experimental group were stained with biotin-conjugated anti-TCR $\alpha\beta$ (mAb H57-597) and then with streptavidin-phycoerythrin and fluorescein isothiocyanate-conjugated anti-CD3 (mAb 145-2C11) and analyzed on a Coulter Epics C flow cytometer as described (8). Percentage of $\alpha\beta^-$ cells among $CD3^+$ cells are presented as histograms. In the cytofluorographs, x and y axes represent green and red fluorescence, respectively, and the z axis represents relative cell number. The left projection of each pair corresponds to the top bar in the indicated set. IFA, incomplete Freund's adjuvant.

heat shock, we investigated the effects of heat shock in combination with the polyclonal activation of T cells by the mitogen Con A. Fig. 3 shows that when heat-shocked cells are polyclonally activated with Con A, the predominance of the $\alpha\beta^+$ lymphocytes among the proliferating T cells is restored to levels identical to those in the non-heat-shocked controls. This indicates that the preferential enrichment of $\gamma\delta^+$ T cells observed in Fig. 2 is not a consequence of the greater thermal resistance of $\gamma\delta$ T cells but is achieved by

selection events induced by heat shock. If this selection is mediated by the TCR, then heat-shocked γ -irradiated bystander cells should be able to induce a selective proliferation of non-heat-shocked $\gamma\delta^+$ T cells.

The result of an experiment designed under the above assumption is presented in Fig. 4. When non-heat-shocked unprimed responder RPLs are cocultured with heat-shocked γ -irradiated syngeneic RPL stimulators, a positive selection of $\gamma\delta^+$ responder T lymphocytes occurs. The proportion of

FIG. 3. Heat shock treatment does not selectively inactivate $\alpha\beta^+$ T cells. Approximately 2×10^5 viable cells were stained with the same reagents and analyzed as in Fig. 2. The percentage of $\alpha\beta^+$ cells among total $CD3^+$ cells are presented as histograms. Both heat-shocked and non-heat-shocked cells of unimmunized mice were cultured in the presence or absence of the T-cell mitogen Con A (5 μ g/ml; Pharmacia). In one experimental group, OVA was added (40 μ g/ml; Sigma).

FIG. 4. Activation of $CD3^+ \alpha\beta^-$ T cells by heat-shocked irradiated bystander splenocytes. RPLs from normal BALB/c mice were prepared as described (10). Syngeneic γ -irradiated (3300 R; 1 R = 0.258 mC/kg) heat-shocked stimulator cells were added in one experimental group, in equal number to the responder cells. Culture conditions, addition of interleukins, and immunofluorescence analysis of the cells selected in culture were performed as for Fig. 2.

$\gamma\delta^+$ T cells represents 88% of the fraction of $\gamma\delta^+$ cells detected when the responder population itself was heat-shocked. Hence, it is not the effect of heat shock on the T cells themselves *per se* that is responsible for their preferential activation.

To ascertain that the $\gamma\delta$ T cells obtained from heat-shocked cultures indeed are proliferating cells and not merely surviving cells, these cells were purified on the fluorescence-activated cell sorter as a $CD3^+ TCR\alpha\beta^-$ population and subjected to cell cycle analysis. Propidium iodide DNA staining of this sorted population (Fig. 5) showed that 66% of the cells have a greater than diploid ($>2N$) DNA content. This proves that the preferential enrichment of $\gamma\delta$ T cells that we have described is due to the active proliferation of these cells subsequent to activation.

Evidence for the selective expansion of a subset of $\gamma\delta$ T cells in the heat shock response comes from a comparison of the composition of the V_γ and V_δ mRNA expressed in the PPD-primed non-heat-shocked ("PPD") and PPD-primed followed by *in vitro* heat-shocked ("HS") RPL populations described in Fig. 2. Total mRNA extracted from purified $\gamma\delta$ T cells from each of these two populations was reverse transcribed into cDNA. The relative abundances of various V_γ and V_δ gene transcripts in each population is assessed using specific J and V gene sequences as primers in the PCRs. Fig. 6 shows that $V_\gamma 4$ is expressed in both PPD and HS populations and so is $V_\gamma 6$. However, from the HS population, less $V_\gamma 6$ RNA is detected compared to $V_\gamma 4$, which is not

the case for PPD cells. No $V_\gamma 1C_\gamma 4$ mRNAs are detected using the 41BNT22 (5) T-cell hybrid as the positive control (data not shown). The two major V_δ genes expressed in PPD cells are $V_\delta 4$ and $V_\delta 5$, while $V_\delta 5$ and $V_\delta 6$ predominates in HS cells. Thus, as a result of heat shock, there is a significant increase in the level of $V_\delta 6$ RNA relative to that of $V_\delta 5$, and a parallel decrease of $V_\delta 4$. It should be noted that $V_\delta 1$ is the only V gene among the set tested that rearranges to both $J_\delta 1$ and $J_\delta 2$. The detailed involvement of each V gene in the heat shock response must await further analysis.

DISCUSSION

The biology of T cells bearing the $TCR\gamma\delta$ is only now beginning to be understood, several years after the isolation of the first γ and δ genes. Surprising features of the γ and δ gene expression have been reported: the sequential programmed rearrangement and expression of V_γ genes during ontogeny (25); the tissue-specific expression of some γ and δ V regions, well-documented for dendritic epithelial cells in the skin (26, 27) and in intraepithelial lymphocytes in the intestinal tract (15, 28, 29); the lack of N-region diversity in some $\gamma\delta$ receptors, contrasting with a significant diversity in others (26–29). The latter observation also appears to observe a strict tissue distribution pattern. These "peculiarities" of the $\gamma\delta$ T cells have not been observed for their $\alpha\beta$ counterparts.

The reactivity of $\gamma\delta$ T lymphocytes seems not to be randomly directed to epitopes similar to those recognized by the large and heterogeneous $\alpha\beta$ T-cell pool. There are documentations that some $\gamma\delta$ T lymphocytes recognize major histocompatibility complex or major histocompatibility complex-like antigens (30–33). This would indicate that the structure of those $\gamma\delta$ receptors allow their reactivity to allogeneic restriction elements in a similar fashion as the $TCR\alpha\beta$. However, the reactivity to TLA or Qa encoded antigens (which was reported for $TCR\alpha\beta$ only in rare situations) seems to indicate that $\gamma\delta$ T cells are preferentially restricted to a subset of major histocompatibility complex antigens (class Ib), usually indifferent for the $TCR\alpha\beta$ and whose biological function is not yet established. Other $\gamma\delta$ T cells react with mycobacterial antigens of *Mycobacterium leprae* or *M. tuberculosis*. Among such antigens, the 65-kDa heat shock protein hsp65, a heat shock protein well-conserved in phylogeny, appears to stimulate both murine and human $\gamma\delta$ T cells (5–8). It is not surprising that $\gamma\delta$ RPLs are activated *in vivo* upon exposure to PPD aerosols and can be further expanded *in vitro*, considering that the lung is an elective place for tuberculosis development.

The data presented in this report indicate that $\gamma\delta$ lymphocytes are stimulated and can be preferentially enriched in heterogeneous cellular populations exposed to heat shock. Moreover, mycobacterial antigens from CFA can act to prime $\gamma\delta$ T cells in the lymph nodes *in vivo*, and these cells can be further triggered *in vitro* by heat shock-induced stress proteins. In addition, *in vivo* exposure of RPLs to PPD,

FIG. 5. Cell cycle analysis of $\gamma\delta^+$ T cells in heat-shocked cultures. $\gamma\delta^+$ ($CD3^+ \alpha\beta^-$) cells were purified on the fluorescence-activated cell sorter from day 6 cultures of lymph node lymphocytes, primed *in vivo* with CFA, and exposed to heat shock *in vitro* as exemplified in Fig. 2. The DNA content of propidium iodide-stained nuclei was estimated by flow cytometry. Cells in G_0 and G_1 are in the 2N fraction, and cells in cycle (S, G_2 , and M) have $>2N$ DNA content. In the total viable cell population, from which the $\gamma\delta$ cells were isolated, cells with $>2N$ DNA content represented 55% (data not shown). N is the haploid amount of DNA.

FIG. 6. A comparison of V gene usage between $\gamma\delta^+$ RPLs primed *in vivo* with PPD and expanded without heat shock (PPD cells) or with heat shock (HS cells). Cells were prepared as for Fig. 2, and RNA preparations and PCR analysis were performed. A 5- μ l sample of each reaction mixture was analyzed on a 1.5% agarose gel. The PCR products are all of the expected sizes. pBR322 digested with *Alu* I serves as the molecular size standard (base pair).

followed by heat shock, led to a proliferating population of T lymphocytes, among which the $\gamma\delta$ T cells represent >50%. Cell cycle analysis demonstrate that the $\gamma\delta$ T cells selected *in vitro* in such experiments are actively proliferating. Furthermore, their induction could also be achieved by coculturing with heat-shocked syngeneic bystander cells.

The analysis of the γ and δ V and J gene segments expressed in the enriched $CD3^+ \alpha\beta^-$ T cells demonstrate that a significant fraction of the $\gamma\delta^+$ T cells initially stimulated by mycobacterial antigens are further expanded subsequent to heat shock. There are some quantitative differences in the expression of some V γ and V δ genes between the mRNA extracted from the PPD and HS populations derived from RPLs as revealed in the PCR assays. These data indicate that heat-induced murine stress proteins trigger a distinct subset of $\gamma\delta$ T cells within the pool of $\gamma\delta$ T cells that was activated with PPD and, therefore, demonstrate that the response to bacterial antigens and host stress proteins do not involve completely overlapping sets of the TCR $\gamma\delta$.

As shown in this paper, both lymph node and pulmonary $\gamma\delta$ T cells can respond to heat-induced stress proteins. $\gamma\delta$ T cells from both sources have also been shown to respond to *M. tuberculosis* antigen by *in vivo* priming and *in vitro* expansion. Considering the difficulties in demonstrating $\gamma\delta$ T-cell reactivity to standard nominal antigens, the apparent ease with which $\gamma\delta$ reactivity to mycobacterial antigens and self heat-shocked proteins can be demonstrated implies that a large fraction of the $\gamma\delta$ T-cell repertoire is directed against stress proteins of both bacterial and murine origin.

The data reported here suggest an extensive cross antigenicity of prokaryotic heat shock proteins and murine stress proteins, as defined by $\gamma\delta$ T-cell recognition. A dual role of such lymphocytes should now be considered. On one hand, they could mount a specific defense against pathogens, whereas, on the other hand, their reactivity to self stress proteins could allow them to initiate or amplify immune reactions localized at the site of aggression.

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