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Complex network of cytokines activating murine microglial cell activity against *Acanthamoeba castellani*



Abstract

In the central nervous system, cytokine-primed microglia play a pivotal role in host defence against *Acanthamoeba castellani* infections. In this study, the effect of rIL-1beta, rIL-6 or rTNF-alpha, combined or not with rIFN-gamma, on *A. castellani* infection of murine microglia was examined. Priming of microglial cells with either rIL-1beta or rIL-6, in the presence or absence of rIFN-gamma,

triggered amebastatic activity, while the treatment of microglia with rTNF-alpha plus rIFN-gamma additively triggered, in a dose-dependent fashion, amebicidal activity. Inasmuch as NGMA affected cytokine-triggered anti-parasitic activity during the priming process, the NO-dependent pathway itself appears not to be directly involved in the anti-amebic capacities. These data suggest that the proinflammatory cytokines IL-1beta, IL-6 or TNF-alpha could trigger anti-microbial activity against *A. castellanii* infection in the brain.

Full text

INTRODUCTION

Acanthamoeba species are pathogenic, opportunistic, free-living amebas [1, 2] capable of infecting the eye, skin and brain [2-9] of human beings and other animals. *Acanthamoeba* spp. exist in cyst and trophozoite stages in the environment. In humans, the cysts are potentially infectious because of their capacity to convert to the trophozoite phase, but only the trophozoites are invasive [10]. The invasive capability depends on ability of the parasite to adhere to mucosal surfaces, migrate through tissues, and release proteases that can destroy connective tissue [11]. Moreover, *A. castellanii*, a weak pathogenic species [12, 13], can also induce, by a 136-kDa mannose-binding trophozoite protein [14], either cytolysis or apoptosis of target cells *in vitro* [15-17]. *A. castellanii* infection generally induces keratitis in healthy individuals [3, 4]; however, it causes fatal granulomatous amebic encephalitis (GAE), and cutaneous or disseminated acanthamebiasis in immunocompromised patients, including those with the acquired immunodeficiency syndrome (AIDS) [6-9, 18, 19]. GAE is characterized by focal granulomatous lesions in the central nervous system (CNS) resulting from an immunological response by the host to the presence of amebas. The role of the somatic immune system in host defence against *Acanthamoeba* spp. is well known. Phagocytes, cytokines, antibodies and complement appear to be the first-line defence against *Acanthamoeba* infections in humans [10, 20-23]. In particular, macrophages primed with interferon (IFN)-gamma or immune serum prevent *A. castellanii* replication [21]; moreover, macrophages activated with tumor necrosis factor (TNF)-alpha, interleukin (IL)-1alpha or IL-1beta cytokines exhibit an amebolytic capability for *Naegleria fowleri* [24] but not for *Acanthamoeba* spp. [13]. The cell types and mediators involved in host defence within the CNS, however, have not been clearly defined. Recent *in vitro* studies, to elucidate the role of glial cells, such as microglia, in host defence against *Acanthamoeba* spp. have been carried out

using enriched rat microglia challenged with these parasites [25, 26]. Microglia, the resident macrophages of the CNS [27], form a neuron-glia network with potential macrophage-like functions that may be related to their host defence role, including expression of major histocompatibility complex class II Ag (MHC II Ag) [28], intracellular adhesion molecule 1 (ICAM-1) [29], phagocytosis of microorganisms, such as *A. castellani* and *T. gondii* [25, 29-32], and production of cytokines upon stimulation [25, 29-36], and free radicals (*i.e* nitric oxide (NO)) [29, 31]; however, microglial cells may also contribute to neuronal injury [37] or be induced to apoptotic death after challenge with *A. culbertsoni* [26]. On the other hand, our previous studies and others have shown that murine microglia are suitable host cells for *T. gondii* but, when primed by TNF-alpha and prolactin hormone (PRL) or IFN-gamma plus endotoxin (lipopolysaccharide, LPS) are able to inhibit parasite growth *via* an NO-dependent pathway [29, 31]. Conversely, microglia derived from human fetuses, activated by TNF-alpha or IL-6, in the absence or presence of IFN-gamma, appear to inhibit *T. gondii* replication through an NO-independent mechanism [32]. Since microglia and proinflammatory cytokines could be involved in the immunopathogenesis of GAE, the aim of this study was to investigate the effect of IL-1beta, IL-6 or TNF-alpha on *A. castellani* growth in murine microglia, and to precise the understanding of the role of IFN-gamma in host defence against this parasite in the CNS.

MATERIALS AND METHODS

Reagents

Recombinant (r) mouse IFN-gamma (rIFN-gamma), rTNF-alpha, rIL-6 and rIL-1beta were purchased from Genzyme (MA, USA). LPS (*E. coli* O128:B12) and superoxide dismutase (SOD) were obtained from Sigma. N^G-monomethyl-L-arginine (N^GMA) was purchased from Amersham.

The presence of endotoxin in reagents was checked by the *Limulus ameobocyte* lysate test [38] and all results were negative (*i.e* < 0.05 EU of LPS/ml of sample).

Microglial cell (MGC) cultures

Cell cultures were prepared as previously described by Chao *et al.* [30]. Briefly, cerebral cortical cells, from 1-day-old BALB/c mice, were dissociated after a 30

min trypsinization (0.25%), and plated in 75-cm³ Falcon culture flasks (Nunc, Denmark) in RPMI 1640 medium (Seromed), containing 20% heat-inactivated fetal calf serum (FCS; Seromed), 2 mM L-glutamine (Seromed), 20 mM HEPES (Seromed), 100 U/ml penicillin (Sigma) and 100 mg/ml streptomycin (Sigma). The medium was replenished 1 and 4 days after plating. On day 8 of culture, plates were shaken for 20 min, at a speed of 200 rpm in an orbital shaker to remove oligodendrocytes. On day 12 of culture, plates were shaken again for 2 hours at a speed of 180 to 200 rpm. Harvested cells were filtered through a 20 µm nylon mesh, plated onto a 60 mm Petri dish and incubated for 15 min at 37° C. After extensive washing with culture medium, adherent cells (microglia) were collected with a rubber policeman and centrifuged at 200 g for 10 min. Isolated microglia were plated onto glass cover slips previously coated with poly-L-lysine (Sigma) and placed in wells of 24-well plates. Purified microglia were composed of a cell population, > 98% of which stained with MAC-1 antibodies (Boehringer-Mannheim, Biochemical, Milan, Italy) and < 2% stained with antibodies specific to glial fibrillary acid protein (Sigma), an astrocyte marker.

Preparation of amebas

A. castellani (ATCC 30010) was obtained from the American Type Culture Collection. Parasites were grown as axenic cultures in peptone-yeast-glucose solution in tubes at 35°C, as previously described [39]. For the infection of MGC, the parasite suspension was removed from a culture of *A. castellani* in the log growth phase and then centrifuged at 250 g for 10 min. The pellet containing free trophozoites was then resuspended with RPMI 1640 medium.

Effects of rIL-beta, rIL-6 or rTNF-alpha on A. castellani growth in MGC

In preliminary experiments, MGC, at different host cell/parasite ratios, were incubated (37° C, in 5% CO₂) with medium alone (negative control (NC)) or containing different concentrations of LPS or rIFN-gamma, alone or together, at various times. The host cell/parasite ratios, concentrations and optimal times turned out to be about 5:1 to 10:1, 10-100 ng/ml and 24-48 hours for LPS, and 5:1 to 20:1, 100-500 U/ml and 24 hours for rIFN-gamma. Inasmuch as greater parasitostatic capacity was observed when 10 ng/ml LPS were combined with 100 U/ml rIFN-gamma, and which was also in agreement with other studies [30], we considered this combination as the positive control (PC). The lower limit

concentration of rIFN-gamma was then used in our subsequent experiments for microglia priming.

To investigate whether rIL-beta, rIL-6 or rTNF-alpha could trigger anti-parasitic activity in microglia primed or not with rIFN-gamma, after the addition of 100 U/ml SOD (an O_2^- scavenger), MGC cultures (5×10^4 cells/well) were treated *without or with* various doses of rIL-beta, rIL-6 or rTNF-alpha in the absence or presence of 100 U/ml of rIFN-gamma. After incubation for 24 hours under normal culture conditions, the cells were extensively washed with prewarmed, phosphate - buffered saline (PBS) and reincubated for an additional 1 hour under the same culture conditions, in a medium containing 5×10^3 living *A. castellanii* trophozoites, followed by three washes with warm PBS to remove non-ingested parasites. The cells of one cover slip were fixed in methanol and marked with Diff Quik (Baxter Scientific Products, McGaw Park, IL, USA) stain, for determination of the number ingested trophozoites per 100 MGC at the start (time 0) of the assay. Other replicates were incubated in the presence of SOD and fresh medium with or without LPS plus rIFN-gamma or specific cytokine, combined or not with rIFN-gamma. After 20 hours (time 20) under normal culture conditions, the cells were fixed and stained as above. The course of intramicroglial infection was assessed microscopically by counting the number of amebas/100 cells in Diff Quik stained preparations. If the number of parasites per 100 MGC at time 20 of the assay had increased by more than 7% compared to the number of parasites per 100 MGC at the start of the assay, the results were discarded. Intramicroglial growth, an index of either parasitostatic or parasitocidal activity, was expressed as fold increase (FI), *i.e.*, the ratio of the mean number of parasites per 100 MGC after 20 hours (T20) of infection and the mean number of parasites per 100 MGC at 1 hour (T0) of the assay [29]. All the FI values higher than 1.0 ($FI > 1.0$), but significant as compared to control, indicate parasitostatic activity, and an FI value equal to 1.0 ($FI = 1.0$), corresponding to the absence of parasite growth, indicates the highest parasitostatic capacity. Whereas, all the FI values lower than 1.0 ($FI < 1.0$) indicate parasitocidal activity, an FI value equal to 0 ($FI = 0$), corresponding to total parasite destruction, indicates the highest parasitocidal activity. Therefore, here, all the $FI \geq 1.0$ or < 1.0 express amebastatic or amebicidal capacity respectively, and the highest FI value albeit smaller than 1.0, corresponds to the limit capacity between the maximal parasitostatic and the minimal parasitocidal capacity, and indicates the cut-off of amebastatic versus amebicidal activity.

Cytotoxicity assays

The ability of microglia primed with rTNF-alpha plus rIFN-gamma to destroy *A. castellani*, was also assessed by measuring release of [5, 6 ³H]-uridine from parasites in the presence of activated microglia as previously described [13, 21]. Briefly, each well of a 24-well plate containing MGC (5 x 10⁴) received different doses of rTNF-alpha in the absence or presence of 100 U/ml of rIFN-gamma. After incubation for 24 hours at 37° C in 5% CO₂, the cells were extensively washed with PBS and cocultured with 5 x 10³ radiolabeled (10 mCi/ml of [5, 6 ³H]-uridine; Amersham International plc, UK) trophozoites for 20 hours under normal culture conditions. Following coincubation, microtiter plates were centrifuged for 10 min at 1 000 g. Supernatant fluids were removed individually, and the counts per minute (CPM), an index of cytolysis, were measured by liquid scintillation spectrometry. Controls consisted of wells containing MGC in medium or in LPS (10 ng/ml) plus rIFN-gamma (100 U/ml), with or without exposure to trophozoites, and trophozoites in medium alone. One set of these controls was checked for both spontaneous and total lysis. The values of spontaneous release were obtained by quantifying the CPM present in the supernatant fluids from wells containing [5, 6 ³H]-uridine-labeled amebas in medium. Whereas the values of maximum release were achieved by measuring the CPM in both the supernatant fluid and the lysed cellular pellet from wells containing radiolabeled amebas in medium. All the total lyses were accomplished by repeated freezing and thawing of trophozoites (5 times at -85°C; complete lysis was confirmed microscopically).

The percentage of specific release of radiolabel correlating to cytolysis of amebas was calculated according to the following formula:

$$\% \text{ Specific release} = \frac{[\text{cpm (unknown supernatant)} - \text{cpm (spontaneous release)}]}{[\text{cpm (maximum release)} - \text{cpm (spontaneous release)}]} \times 100$$

Measurement of NO production by primed MGC and correlation with anti-amebic activities

NO generation was evaluated by measuring nitrite (NO₂⁻) accumulation in the culture supernatants of MGC incubated for 24 hours with medium alone or containing LPS (10 ng/ml) plus rIFN-gamma (100 U/ml) or rTNF-alpha (100 U/ml)

plus rIFN-gamma (100 U/ml), before challenge with *A. castellani*, and in the culture supernatants collected during the 20 hours of infection, by the Griess colorimetric reaction, as previously described [40]. Here, the measurement of NO_2^- micromoles for 5×10^4 cells was taken as a reflection of NO production.

To investigate the implication of NO in the induction of anti-parasitic activity, 300 μM N^GMA (a competitive inhibitor of L-arginine) were added immediately, during the 24 hour incubation period of the MGC with rTNF-alpha plus rIFN-gamma, before infection (priming process), and during the 20 hour challenge period with *A. castellani* (infection process).

Statistical analysis

Each set of results are presented as the mean $\pm$ standard deviation (SD) of triplicate independent measurements of four, separate repeated experiments. Differences were considered to be significant if $p < 0.05$, as determined by the Student's *t*-test.

RESULTS

Effect of rIL-beta, rIL-6 or rTNF-alpha on A. castellani growth in MGC

To evaluate whether rIL-1beta, rIL-6 or rTNF-alpha could trigger anti-parasitic activity, MGC were primed with each of the above-mentioned cytokines at different doses, in the absence or presence of 100 U/ml IFN-gamma for 24 hours, before infection with *A. castellani*. Figure 1A shows that MGC primed with either 0.03 or 20 U/ml rIL-1beta alone did not develop anti-parasitic effects ($p = \text{ns}$ or $p < 0.01$ each *versus* negative control or positive control, respectively). A significant amebastatic effect was then seen when MGC were primed with either 100 or 500 U/ml IL-1beta in the absence or presence of 100 U/ml IFN-gamma ($p < 0.05$ each *versus* NC or PC, respectively). Similarly (Figure 1B) to IL-1beta, no parasitostatic effect was triggered with 0.03 or 20 U/ml IL-6 alone ($p = \text{ns}$ or $p < 0.01$ each *versus* NC or PC, respectively), whereas a slight amebastatic effect was induced with 100 or 500 U/ml IL-6 ($p < 0.05$ each *versus* NC or PC, respectively). However, maximal amebastatic effects, in a dose-dependent fashion, were then triggered with either 100 or 500 U/ml rIL-6 combined with 100 U/ml IFN-gamma ($p < 0.01$ or $p = \text{ns}$ each *versus* NC or PC, respectively). Analogously (Figure 2A) to rIL-1beta and IL-6, no anti-amebic effect was induced

with 0.03 or 20 U/ml rTNF-alpha alone ($p = \text{ns}$ or $p < 0.01$ each *versus* NC or PC, respectively), while a low amebastatic effect was triggered with 100 or 500 U/ml rTNF-alpha ($p < 0.05$ each *versus* NC or PC, respectively). An amebicidal effect was then induced when 100 or 500 U/ml rTNF-alpha were added together with 100 U/ml rIFN-gamma ($\text{FI} = 0.7 \pm 0.3$ or 0.8 ± 0.5 each *versus* 7.6 ± 1.0 in NC or 1.7 ± 0.3 in PC, $p < 0.001$ or $p < 0.05$, respectively). This effect appeared to be additive and dose-dependent. Furthermore, the presence of polymyxin B added to bind free endotoxin, did not reverse amebicidal activity; in fact, the values obtained from MGC treated with rTNF-alpha (100 U/ml) and rIFN-gamma (100 U/ml) plus 30 mug/ml polymyxin B were similar ($\text{FI} = 0.9 \pm 0.2$) to those in MGC primed with rTNF-alpha (100 U/ml) and rIFN-gamma (100 U/ml) alone. Visual observation of the microglial cultures using an inverted light microscope did not reveal cystic forms, but only ingested trophozoites, which resulted in round rather than ameboid forms, with punctate lesions. To confirm the visual results, destruction of amebas was quantified using [^3H]-uridine-labeled amebas in a cytotoxicity assay. Our findings (Figure 2B) showed that MGC, primed with either 0.03 or 20 U/ml rTNF-alpha alone, did not cause a specific release of radiolabel from amebas ($p = \text{ns}$ or $p < 0.01$ each *versus* NC or PC, respectively), whereas MGC, activated with either 100 or 500 U/ml rTNF-alpha, induced a 30 ± 1.1 or $28 \pm 1.0\%$ specific release ($p < 0.05$ each *versus* NC or PC, respectively). However, a maximal specific release by either 70 ± 0.9 or $67 \pm 0.8\%$ was then elicited with 100 or 500 U/ml rTNF-alpha in the presence of 100 U/ml rIFN-gamma ($p < 0.001$ or $p < 0.05$ each *versus* NC or PC, respectively). These values corresponded well contrasted with the FI presented in Figure 1A.

NO production by primed MGC and correlation with anti-parasitic activity

To determine if cytokine priming induced the generation of NO by MGC, both at 24 hours of the priming process with rTNF-alpha (100 U/ml) plus rIFN-gamma (100 U/ml), and at 20 hours of the infection process, supernatant media were saved and analysed spectrophotometrically. The results (Figure 3A and 3B) showed that in both cases, the priming of microglia with rTNF-alpha plus rIFN-gamma elicited NO production by these cells. At 24 hours of the priming process (Figure 3A), the nitrite level was $48.0 \mu\text{M} \pm 11.1$ ($p < 0.001$ or $p = \text{ns}$ *versus* negative control or positive control, respectively), while at 20 hours of the infection process (Figure 3B) it was 24.7 ± 6.0 ($p < 0.01$ or $p = \text{ns}$ *versus* NC or PC, respectively).

To determine the implication of NO in the induction of anti-parasitic activity, MGC were treated with rTNF-alpha (100 U/ml) plus rIFN-gamma (100 U/ml) in the presence of N^GMA added during the 24 hour period of the priming process, before challenge with *A. castellanii*, and during the 20 hour period of the infection process. We found that the presence of N^GMA during the priming process of MGC (3A and 3C), blocked both the production of NO (nitrite = $8.3 \mu\text{M} \pm 1.0$, $p = \text{ns}$ or $p < 0.001$ *versus* NC or PC) and anti-parasitic activity (FI = 7.2 ± 1.0 , $p = \text{ns}$ or $p < 0.001$ *versus* NC or PC respectively); while, the presence of N^GMA during the infection process (3B and 3D), completely abolished the generation of NO (nitrite = $5.0 \mu\text{M} \pm 1.1$, $p = \text{ns}$ or $p < 0.01$ *versus* NC or PC respectively), but did not alter the anti-amebic capacity (FI = 0.8 ± 0.3 , $p < 0.001$ or $p < 0.05$ *versus* NC or PC respectively). These data suggest that NO itself does not directly influence the anti-amebic functions.

DISCUSSION

Cytokines such as IFN-gamma, TNF-alpha, IL-1 and IL-6, as well as PRL and LPS are known to be important mediators of cellular resistance against parasites. The importance of cytokines and microglia in regulating protective immunity to parasitic infections in the brain is well established [29-32], but their role in cerebral *A. castellanii* infection is not yet well known. Recently, it has been shown that rat microglia have the ability to ingest *A. castellanii* and elicit IL-alpha, IL-beta and TNF-alpha [25]. Based on our previous studies of *T. gondii* replication in murine microglia [29], we presumed that these glial cells and proinflammatory cytokines could be involved in the cerebral anti-amebic activity. From the present study, it can be concluded that low but activating doses of IL-beta, IL-6 or TNF-alpha, alone or in combination with low but activating doses of IFN-gamma, can directly prime murine microglia, and trigger anti-parasitic functions in these cells. This conclusion is based on the following observations. Firstly, rIL-beta combined or not with IFN-gamma, dose-dependently induces a slight amebastatic activity in microglia, accompanied by a decrease in *A. castellanii* growth. Secondly, rIL-6 alone triggers slight parasitostatic activity, whereas combined with rIFN-gamma, optimally prime microglia, in a dose-dependent manner, resulting an effective amebastatic activity, which leads to substantial inhibition of *A. castellanii* replication. Thirdly, rTNF-alpha alone induces a slight parasitostatic activity, whereas together with rIFN-gamma it additively triggers, in a dose-dependent fashion, an amebicidal activity that leads to lysis of the parasite. In particular, this destructive capacity is demonstrated by both low FI

values and high percentages of radiolabel release. It is well known that the action of cytokines requires binding of the cytokine to its specific cell surface receptors, which activate the cells. Concentrations of 100 U/ml for rIL-1beta, IL-6, rTNF-alpha or rIFN-gamma are required to prime microglia in order to achieve a significant protective effect, but higher concentrations do not lead to greater effects. It is thus likely that the subsequent lack of dose-response for higher concentrations is linked to the blocking of the specific cytokine receptor. Although IFN-gamma appears to be the major macrophage activator and mediator of resistance to *A. castellanii* and *T. gondii* [21, 41, 42], and IFN-gammaR signalling is the major pathway for the activation of murine microglia [43], there is evidence emerging that rIL-beta, rIL-6 or rTNF-alpha could operate as a key triggering signal in these anti-parasitic activities. It is likely that *in vivo* circulating levels of these cytokines are sufficient to trigger the anti-amebic activities. Because either rIL-6 or rTNF-alpha (but not rIL-1beta) require the presence of rIFN-gamma for an effective amebastatic activity or amebicidal capacity respectively, it is conceivable that binding of rIL-6 or rTNF-alpha to its receptor alone is not sufficient to trigger the intracellular signal transduction pathways implicated in the induction of the anti-amebic capability. Therefore, a second stimulus (*e.g.*, low concentration of rIFN-gamma) is needed for induction of intracellular signalling events that convert the initial interaction of either rIL-6 or rTNF-alpha with its receptor into pathways leading to the biological function. In particular, our findings indicate that IFN-gamma-dependent cytokines are the sole mediators capable of leading either to complete inhibition or destruction of the parasite, whereas the poor inhibition of parasite growth and the lack of a synergistic or additive effect of rIL-1beta in the presence of the rIFN-gamma stimulus, suggest that IFN-gamma-independent cytokines can weakly block the parasite, probably by driving a local inflammatory reaction. Moreover, our results provide strong evidence that TNF-alpha is the most active regulatory factor capable of operating as a key triggering signal in the parasiticidal activity of rIFN-gamma-primed microglia. The amebicidal activity is not due to contamination with LPS as confirmed by the inability to reverse this capacity by treatment with polymyxin B. This activity could be linked to an overexpression of IFN-gammaR associated with high levels of rTNF-alpha in the glial microenvironment. It is conceivable that *in vivo*, at the sites of infection, such amounts of TNF-alpha are produced. The anti-parasitic effects of both TNF-alpha and IL-6 are in agreement with our previous *in vitro* and *in vivo* studies and those of others [29, 32, 44], which have shown that rIFN-gamma, combined with either IL-6 or TNF-alpha [32], or PRL, combined with either IL-6 or TNF-alpha [29], cause a 50, 80, 83 or 90% inhibition of parasite replication in microglia, and TNF-alpha and PRL reduce

the number of cysts and increase survival of mice challenged with *T. gondii* [44]. The mechanism whereby cytokine priming induces anti-parasitic activity in microglia is not yet understood. In both murine macrophages and microglia, the most important effector mechanism directed against *N. fowleri* or *T. gondii* [24, 29, 31] is the NO-dependent pathway *via* the induction of the enzyme NO synthase. Although our data show that the priming effect of rTNF-alpha plus rIFN-gamma on microglia leads to a significant production of NO by these cells, the inhibition of *A. castellanii* growth is found to be NO-dependent only during the priming process, as demonstrated by the ability of N^GMA to completely block the anti-parasitic functions when added during the process of microglial priming, but not when added during the infection process. It is possible that differences in microbial virulence could trigger different anti-parasitic effector pathways. For instance, evidence suggests that during the infection process, NO could affect the anti-amebic functions by triggering other anti-parasitic mechanisms (*e.g.*, the indolamine 2,3-dioxygenase dependent pathway).

CONCLUSION

In conclusion, the findings of this *in vitro* study may be more directly pertinent to understanding the immunopathogenesis of GAE. Such a role for the above-mentioned cytokines in regulating microglial anti-amebic functions *in vivo* is particularly relevant to the control of GAE in immunocompromised subjects. In patients with AIDS, for example, a shift from Th1 to Th2 cytokines, which is accompanied by a down-regulation of IFN-gamma, TNF-alpha, IL-1beta and IL-6 could represent a pivotal event in the development of GAE. Future studies may be carried out with trials involving adding another batch of regulatory factors to infected microglia, aimed at the development of new therapeutic strategies that may be used in patients at risk of cerebral acanthamebiasis.

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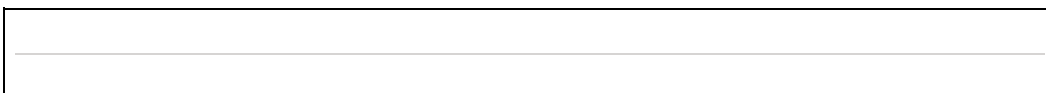
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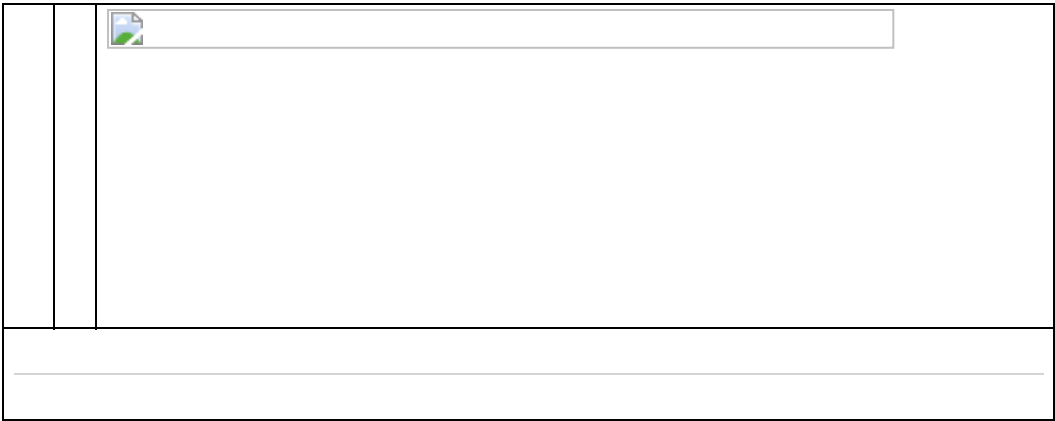
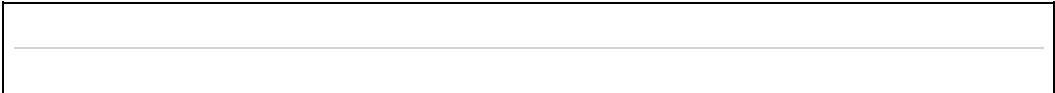
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