

EXPOSURE TO HEPATITIS C VIRUS INDUCES CELLULAR IMMUNE RESPONSES WITHOUT DETECTABLE VIREMIA OR SEROCONVERSION

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Abstract. Sporadic cases of cell-mediated immunity (CMI) in persons exposed to hepatitis C (HCV) but evidently uninfected have been reported. To further define this, we measured CMI in individuals without evidence of HCV infection, that is, negative for HCV-antibodies (anti-HCV) and RNA, residing in a rural Egyptian community where prevalence of anti-HCV was 24%. Cell-mediated immunity (CMI) measured by interferon-gamma (IFN- γ) enzyme-linked immunospot (ELISPOT) assay, confirmed by intracellular staining using flow cytometry, against HCV peptides was measured in seronegative individuals with high-risk (HR) and low-risk (LR) exposures to HCV. Thirteen of 71 (18.3%) HR subjects but only 1 of 35 (2.9%) LR subjects had detectable CMI ($P = 0.032$). These data are compatible with the hypothesis that exposures to HCV may lead to development of HCV-specific CMI without anti-HCV and ongoing viral replication. We speculate induced CMI clears HCV sometimes when anti-HCV is not detectable, and HCV-specific CMI is a useful surrogate marker for exposure to HCV.

INTRODUCTION

Hepatitis C virus (HCV) is one of the most frequent causes of liver disease with an estimated 170 million being chronically infected. Hepatitis C virus (HCV) infection is most often diagnosed by detecting virus-specific antibodies (anti-HCV); 75–80% of those having anti-HCV also have active infections with viremia, marked by the presence of HCV-RNA. Approximately 80% of infected individuals develop chronic hepatitis, among whom 20–30% may progress to hepatic cirrhosis and 2–3% of these go on each year to have hepatic failure and/or hepatocellular carcinoma.^{1,2}

Strong and persistent cell-mediated immune responses have been reported in HCV-seronegative individuals with documented exposure to HCV in the absence of detectable viral RNA,^{3–8} and healthy family members persistently exposed to chronically HCV-infected patients displayed immunologic memory, presumably established by a subclinical infection.⁹ Furthermore, chimpanzees challenged with low doses of HCV did not develop detectable viremia or anti-HCV, yet they had early and broad CD4⁺ and CD8⁺ T-cell responses to multiple HCV epitopes.¹⁰

HCV is the most common cause of chronic liver disease in Egypt, where prevalence of anti-HCV is 10-fold greater than in the United States and Europe.^{11–14} The most consistent risk for incident (new) HCV infections in a rural village with an overall anti-HCV rate of 24% was living in a household with more than one other person having anti-HCV.¹³ Therefore, we studied HCV-specific cell-mediated immunity (CMI) in individuals with high-risk (HR) and low-risk (LR) exposures to HCV from this community to test the hypothesis that exposures to presumably lower concentrations of the virus that may occur in household contacts could stimulate HCV-

specific CMI with subclinical resolving infection in the absence of detectable anti-HCV or HCV-RNA.

MATERIALS AND METHODS

Subjects and statistical analysis. The subjects were recruited from a cohort of 3,888 individuals over the age of 5 randomly selected by households from a community of 11,000 inhabitants in the Nile delta.^{12,13} A community-wide anti-HCV prevalence of 24.3% was ascertained based on a cross-sectional survey in 1999. We revisited this population in 2003 to collect additional information and blood samples from 175 anti-HCV and HCV-PCR negative subjects who lived in HR and LR households (Table 1). When retested, 15 (8.6%) of these individuals had seroconverted to anti-HCV during the 4 years, and 54 participants had samples that were either insufficient or not tested for CMI responses, leaving 106 subjects in our study population. The 71 HR HCV-negative participants were those who had at least two household members with anti-HCV, whereas the 35 LR HCV-negative participants had no household members who had anti-HCV. The Egyptian Ministry of Health and Population and the University of Maryland Institutional Review Boards reviewed and approved the protocol and consent forms. Informed consent was obtained from all participants. Fisher's exact test was used for data analysis.

Sample collection. Peripheral blood mononuclear cells (PBMCs) were purified by Ficoll-Hypaque before being cryopreserved in the presence of 20% autologous serum and 10% dimethyl sulfoxide (DMSO) in liquid nitrogen until analyzed. Before performing any assay, PBMCs were thawed, washed three times, and counted for viability. Aliquots of sera were frozen and stored at -70°C until examined. Because the frozen cells were subjected to freezing, transportation, and thawing that could affect the viability of cells from vial to vial, results following phytohemagglutinin (PHA) stimulation of the thawed cells evaluated the viability of the cryopreserved PBMCs. The average number of interferon- γ secreting cells (ISCs)/10⁶ cells in 25 subjects examined by enzyme-linked

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TABLE 1

Characteristics and immune responses in subjects who were anti-HCV and HCV-RNA negative from low-risk and high-risk households

Characteristics	Low-risk household	High-risk household
Number screened	48	127
Number having CMI tests	35	71
CMI positive	1 (2.9%)	13 (18.3%)
Mean age in 2003	21	23
Male	15 (42.9%)	41 (57.7%)
Position in household		
Father	5	1
Mother	10	11 (1 CMI positive; 9%)
Son	10 (1 CMI positive; 10%)	40 (8 CMI positive; 20%)
Daughter	10	19 (4 CMI positive; 21%)

HCV, hepatitis C virus; CMI, cell-mediated immunity.

immunospot (ELISPOT) after PHA stimulation was 5,148 ± 1241. Results of tests upon thawed PBMCs having less than the mean - 2SD (i.e., less than 2,500) for ISCs/10⁶ cells after PHA stimulation were excluded from analysis.

HCV-RNA measurements. Sera were tested for HCV-RNA using reverse-transcription polymerase chain reaction (RT-PCR) according to Abdel-Hamid and others.¹⁵ Sera that were negative by this direct method were retested after RNA extraction using the QIAmp Viral RNA Kit (QIAGEN, Santa Clara, CA). Aliquots of 140 µL of sera were used according to the manufacturer's instructions. From 60-µL extract, 55 µL of purified RNA were used for each RT-PCR reaction.

Antibody testing. Quantitative measurement of anti-HCV antibody response was performed using Ortho HCV 3.0 EIA (enzyme immunoassay) test system (Ortho Diagnostic System, Raritan, NJ) according to the manufacturer's instructions, and the cutoff values were calculated.

HCV peptides and ELISPOT assay. The breadth and magnitude of HCV-specific T-cell responses *ex vivo* in PBMCs were measured by a quantitative and functional IFN-γ ELISPOT assay. To avoid bias toward pre-selected epitopes and human leukocyte antigen (HLA) alleles, we used as a source of antigen a panel of 216 peptides covering about 72% of the HCV polyprotein spanning the Core and NS3-NS5B region. All peptides were synthesized with free N-terminal amine and free C-terminal carboxyl groups, using standard Fmoc solid phase methods,¹⁶ and were purified by preparative high-performance liquid chromatography (HPLC). The peptides, 20 amino acids (aa) in length and overlapping by 10 aa, were reconstituted in 100% DMSO at 20 mg/mL and used in the assay at final concentration of 5 µg/mL per peptide. The peptide sequence, spanning the Core and the NS3-NS5B region, reproduced the aa sequence of the HCV BK strain.¹⁷ To facilitate the analysis, the 216 peptides were combined in seven pools covering Core, NS3 protease (NS3p), NS3 helicase (NS3h), NS4, NS5a, and NS5b (split in two pools NS5bI and NS5bII).

IFN-γ ELISPOT assay was performed as previously described.¹⁸ To estimate the number of HCV-specific ISCs, *ex vivo* unexpanded PBMCs were added at different concentrations (1 × 10⁵ to 2 × 10⁵ cells/well) in 100 µL of complete RPMI supplemented with 10% heat-inactivated human AB serum. Peptides were then added at 5 µg/mL final concentration. Concanavalin A (Con-A) or PHA and DMSO served, respectively, as positive and negative controls. The plates were developed as described,¹⁸ and the number of spots was

counted by an automated ELISPOT reader system (Bio-reader 3000 LC; BioSys GmbH, Karben, Germany). The response was considered positive when the following conditions were met: IFN-γ production present in PHA or Con-A stimulated wells > 2500 ISCs/million PBMCs; number of ISCs/10⁶ PBMCs to at least one HCV peptide pool was greater than 55; number of ISCs in positive wells was three times the number detected in DMSO mock control wells; and responses were titratable in serial dilutions of PBMCs.¹⁸

Flow cytometric analysis of intracellular IFN-γ secretion by HCV-specific T cells. To characterize the phenotypes of the T-cells induced in HR individuals, strong CMI responses by ELISPOT assay were further examined by intracellular cytokine staining. PBMCs from 3 HR and 2 LR (as negative controls) were *in vitro* stimulated with the HCV peptide pools as described above, and intracellular IFN-γ production was examined as described previously.¹⁹ PBMCs were washed and stained with surface antibodies, anti-CD4-FITC, CD3-APC, and anti-CD8-PerCP (Becton Dickinson, San Jose, CA) at 4°C for 20 minutes. After the washing, the PBMCs were fixed and permeabilized using FACSPerm Solution (BD Pharmingen, San Jose, CA) for 10 minutes at room temperature, and the Phycoerythrin (PE)-conjugated anti-IFN-γ MAb (BD Pharmingen) was added. Data were analyzed using FlowJo software (Tree Star Inc., San Carlos, CA). The cells were gated on lymphocytes using side and forward light scatter profiles then on CD4⁺ and CD8⁺ cells. The response was considered positive when the following conditions were met: (1) IFN-γ production after stimulation with phorbol myristic acid (PMA) plus ionomycin (positive control) is greater than 10%; (2) the percentage of ISCs is three times the percentage detected in mock stimulated cells plus 2SD; and was calculated to be greater than 0.11%.

RESULTS

Seroconversion and subjects' characteristics. Among the 175 subjects screened, 15 (8.6%) converted from negative to positive for anti-HCV between 1999 and 2003. The 71 HR and 35 LR seronegative subjects tested for CMI were similar in demographic and exposure variables (Table 1). Although there were no statistically significant differences in age, gender, and use of medical care facilities between the groups, HR individuals were slightly older and were more likely to be male and have had prior surgery.

HCV-specific cellular immune responses. Thirteen (18.3%) of the 71 HR individuals had a positive response to at least

one HCV peptide pool, while only 1 (2.9%) among the 35 LR individuals displayed a positive response ($P = 0.032$; Table 1). All but one of those having HCV-specific CMI responses was a child. Prevalence in sons and daughters living in HR household was the same, about 20%. Eleven of the 13 reacting HR subjects responded to one peptide pool, while 1 showed T-cell reactivity against 2 and 1 had a broad response to 4 HCV peptide pools. Cell-mediated immunity to NS4 was present in PBMCs from five subjects, and PBMCs from four reacted to either the NS3 helicase domain or to NS5A. The NS3 protease domain was recognized by PBMCs from two subjects, while CMI was detected against Core and NS5B in PBMCs from one subject each (Figure 1). The magnitude of T-cell responses to individual antigens in subjects from the HR group ranged from 63 to 238 ISCs/ 10^6 PBMCs (median 78). The only positive individual from the LR group had 84 ISCs/ 10^6 PBMCs to NS5A (Figure 1).

To further characterize the cellular responses to HCV, PBMCs from five donors (3 HR and 2 LR) were stimulated with HCV peptide pools. The cells were then gated on lymphocytes using side and forward light scatter profiles then on $CD4^+$ or $CD8^+$ cells (data not shown). Figure 2 shows a representative of flow cytometry data, showing the synthesis of IFN- γ versus CD4 expression stimulated with NS3p (A), and NS5bII (B) peptides pool. PMA and ionomycin were used as a positive control. The data shown are for P46 (a HR donor), who responded to pools NS3p, NS3h, NS4, NS5a, and not NS5bI, and NS5bII by ELISPOT assay (Figure 1). As shown in Figure 2, approximately 0.2% of $CD4^+$ was secreting IFN- γ upon stimulation with NS3p pool peptides. In contrast, no significant responses were seen upon stimulation with NS5bII peptide pools, in agreement with the ELISPOT data. Cells

from the two LR donors and vehicle controls were negative by intracellular staining.

DISCUSSION

Results of our cohort study of incidence and risks for HCV infection in the same village are supported by the current study showing that 8.6% of HCV-negative individuals seroconverted to anti-HCV during the 4-year period of observation.¹³ Difference in exposures to HCV in individuals living in the same community allows testing the hypothesis that HCV-specific T-cell responses can be biomarkers for prior HCV exposure and recovery. This is supported by our data showing 18% of the HR family members (all but 1 of 13 being children) not having HCV antibodies or RNA had CMI to HCV antigens, in contrast to only 1 (3%) of those from LR households.

Previous studies showed HCV-specific CMI can be detected in individuals who resolve infection but very rarely in chronically infected patients, supporting the view that specific HCV-CMI has a major role in control of the infection.^{5,6,20-23} During a prospective study of acutely infected individuals, we discovered that HCV-specific CMI was more frequently detected during acute HCV infection than in patients with chronic persistent infection.¹⁸ Furthermore, a significantly broader T-cell response was present in patients with self-limiting HCV infection than in those whose infection became chronic. Therefore, CMI appears to be an immune correlate of a benign course of infection.

Although the intracellular staining signals induced by HCV stimulation were weak in comparison to the ELISPOT re-

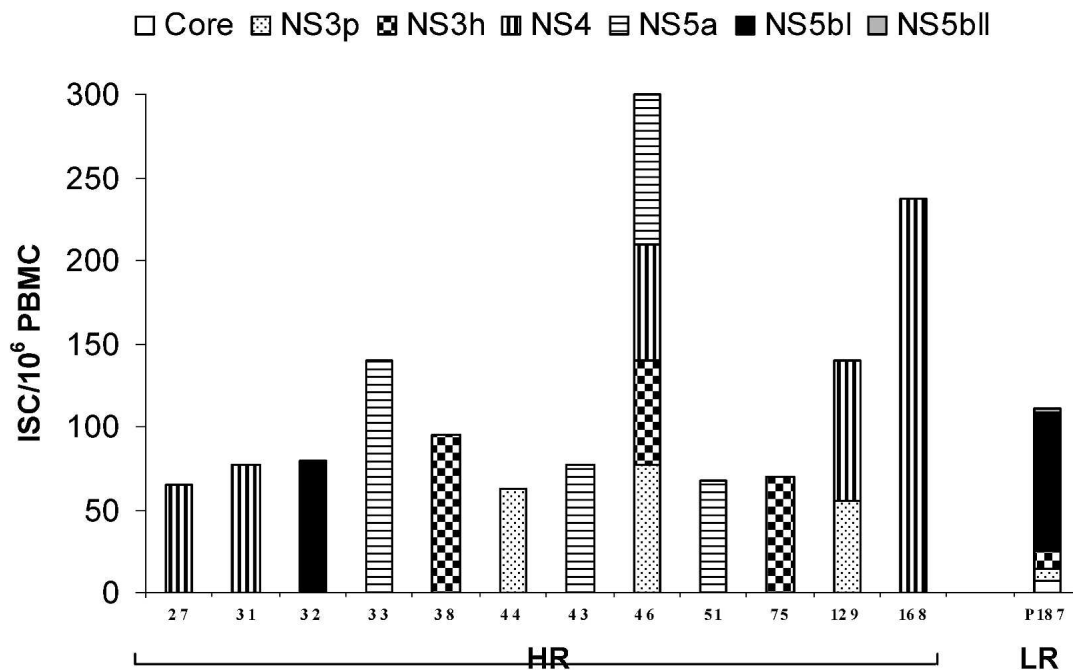


FIGURE 1. Magnitude and breadth of the T-cell response against individual HCV antigens was measured in PBMCs from HR and LR subjects by IFN- γ ELISPOT assay. Individual HR and LR subjects are indicated on the horizontal axis. Each bar represents the number of IFN- γ secreting cells (ISCs)/ 10^6 PBMCs in the vertical axis, in response to peptide pools corresponding to Core, NS3 protease (NS3p), NS3 helicase (NS3h), NS4, NS5a, NS5bI, and NS5bII proteins. Only positive responses, as determined by the criteria described in the "Materials and Methods" section, are displayed.

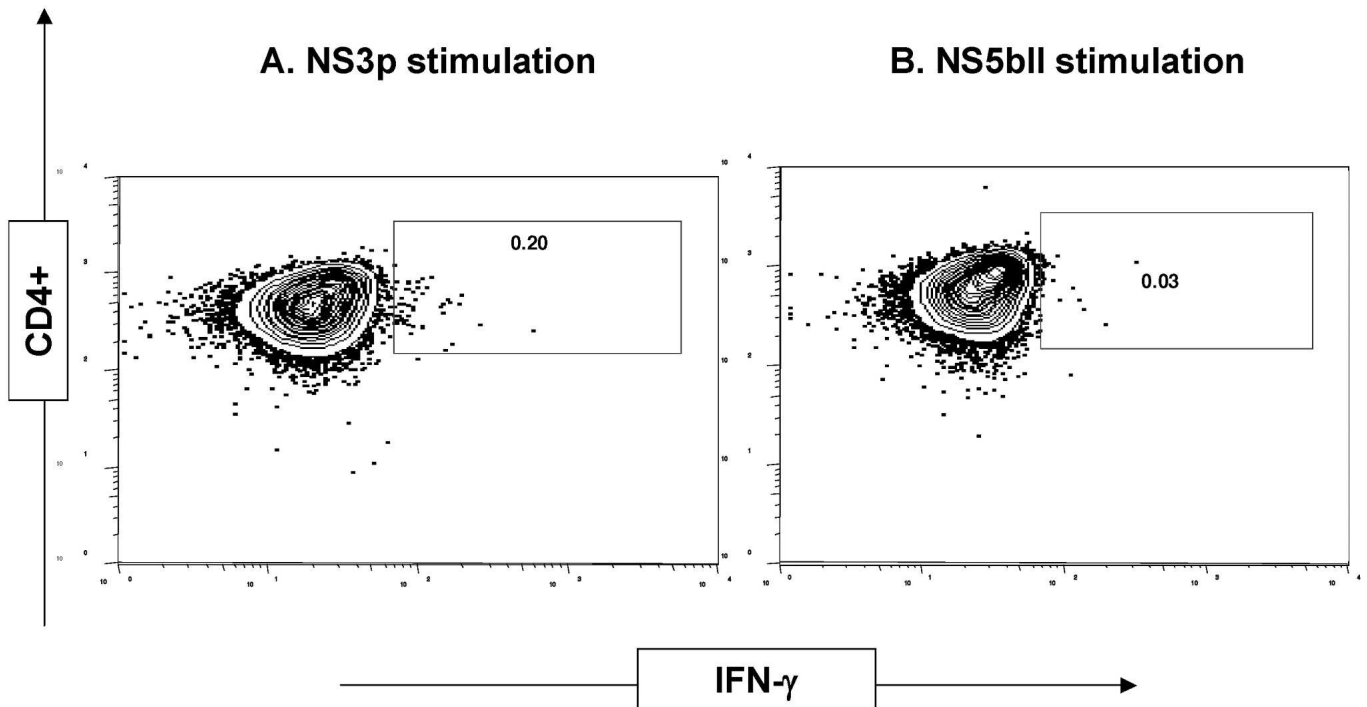


FIGURE 2. Intracellular staining (ICS) for IFN- γ in HCV high-risk donor. T-cell responses against overlapping NS3p and NS5bII pooled peptides were measured in PBMCs from HR P46. PBMCs were stimulated with peptide pools at a concentration of 5 $\mu\text{g}/\text{mL}$ per peptide for a total of 6 hours in the presence of Brefeldin A (10 $\mu\text{g}/\text{mL}$). The cells were then harvested for flow cytometric analysis, and 100,000 events were acquired on a FACS Calibur flow cytometer (BD Pharmingen). Data were analyzed using FlowJo software (Tree Star Inc.). The cells were gated on lymphocytes using side and forward light scatter profiles (not shown), then on CD4 $^{+}$. The figure shows the synthesis of IFN- γ versus CD4 expression in the presence of NS3p (A) or NS5bII (B). The cutoff value was calculated as described in “Materials and Methods.”

sults, as experienced by ourselves and others,¹⁹ they were consistent with the ELISPOT data in the three HR donors examined. The responding cells were mostly CD4 $^{+}$ cells, with borderline or no responses in CD8 $^{+}$ cells (data not shown). The reason for this discrepancy is not clear but may be due to the nature of the peptides used in stimulation: (20-mer) which is optimal for Class II binding and CD4 $^{+}$ stimulation, or the role of these HCV-specific CD4 $^{+}$ T-cells in these HR individuals is more predominant.

Sex-workers with extensive and repeated exposures to HIV, but without detectable viral RNA or antibodies, were shown to develop strong CMI to HIV-antigens.²⁴ The authors suggested that HIV-specific CMI protected these individuals from detectable HIV-infection or led to rapid viral clearance before activation of humoral response could take place. Likewise, some subjects with high risk exposures to HCV (e.g., the household contacts of HCV-infected patients or injection drug abusers) do not develop apparent infection despite repeated exposure to HCV.²⁵ Investigators using assays requiring *in vitro* amplification of T-cell response or an *ex vivo* assay have reported CMI to HCV in seronegative healthy individuals who were either sexual partners of HCV-infected patients, laboratory personnel with potential exposure to the virus, or incarcerated prisoners.^{3,4,7-9} One of our Egyptian colleagues very recently reported in a prospective cohort study detecting HCV-specific CMI in 6 of 8 persistently aviremic and anti-HCV-negative sexual partners of patients with acute HCV hepatitis.²⁶

The mean frequency of HCV-specific ISC measured in our subjects was less than that previously observed during the

acute phase of HCV infection,¹⁸ but is comparable to that measured following recovery.⁶ Also, despite the significant overall conservation of the HCV regions of our test antigens, poor reactivity by the major HCV genotype in Egypt, Genotype 4, to the Genotype 1b peptides used in our ELISPOT assay is a potential problem. However, we have previously demonstrated this same set of 1b peptides can be used to detect HCV-specific T-cell responses in subjects infected by HCV Genotypes 1a, 1b, 2a, 2b, and 3a, with excellent diagnostic sensitivity.¹⁸ Moreover, several studies have reported cross-genotype reactivity for epitopes located within the NS3-NS5 region.^{1,27,28} Therefore, even if there is some underestimation of strength and frequency of CMI responses in the study cohort, our approach remains a viable option to measure and compare T-cell response to HCV exposure.

An explanation for the presence of HCV-specific CMI in otherwise seronegative persons could be that cross-reactive antigens from unrelated organisms primed these T-cell responses. Cross-reactivity of CD4 $^{+}$ T-cells against epitopes of different herpes viruses has been observed in humans,²⁹ and CD8 $^{+}$ T-cell cross-reactivity between non-homologous viruses like influenza A and HCV was also reported.³⁰ However, in this latter study a T-cell response elicited by influenza A cross-reacted with a single epitope from the HCV NS3 protein after repeated *in vitro* re-stimulations with the NS3 peptide. It seems unlikely that the CMI observed in our study would result from T-cell cross-reactivity, because several different HCV proteins stimulated ISC in the *ex vivo* ELISPOT assay without re-stimulation. Thus, we believe these T-cell responses were elicited by exposure to HCV, and the virus

was subsequently cleared leaving an "immunological scar" in the form of antigen-specific CMI.

None of the subjects with HCV-specific CMI responses had a history of hepatitis or symptoms compatible with hepatic disease. The majority of HCV infections are asymptomatic, even during the acute phase.² Therefore, we speculate that our CMI-positive seronegative subjects had a transient very mild infection, possibly associated with low-dose exposure to the virus, which was cleared. Infection is supported by most of the immune responses being to non-structural epitopes of HCV, an indication of replicating virus. A less likely, in our opinion, alternative is the CMI positive individuals have a healthy carrier state with the virus being present in some body compartment (i.e., in the liver) and not in the blood or it is replicating at levels below the sensitivity of our PCR assay (100 copies/mL).

Although our data does not elucidate on how these exposures to HCV occurred, the results suggest they are underestimated in endemic areas using anti-HCV. They could be caused by a common exposure infecting multiple members of the household or they may be between the HCV-infected and non-infected within the household. A common exposure during intravenous therapy of schistosomiasis as occurred in the past¹¹ is not a factor, as all but one of those having CMI in the absence of HCV antibodies were children born after treatment with tartar emetic was discontinued. Other common exposures (e.g., injections given to multiple members of the household using the same needles and syringes) could certainly be a risk. Intra-familial exposures could be many. We speculate they are often low-dose exposures stimulating HCV-specific CMI that aborted, or rapidly cleared, the viremia. These exposures might be from shared toothbrushes or other toilet items. Could they be from exposures to and from cuts, nicks, skin abrasions, and ulcerations that children often have?

Received November 17, 2004. Accepted for publication February 8, 2005.

Acknowledgments: We appreciate the advice of Dr. Paul Hagan and the dedicated assistance of the professional and technical staffs at CFAR, ERDC, and VHRL in Cairo, at the NYBC in New York City, and at the IRBM in Rome in the conduct of these studies. Mar Jan Ostrowski and his administrative staff at the HCP Project in Egypt provided invaluable administrative, financial, and logistic support, and Kelly Weed provided editorial assistance.

Financial support: The research was supported in part by NIH grant RO1-AI47349, the Wellcome Trust-Burroughs Wellcome Fund grants 059113/z/99/a and 059113/z/99/z, and by Italian Ministry of Research (MIUR) grant no. 60470.

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